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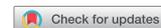


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BRIEF REPORT



Co-dependency between KRAS addiction and ARHGEF2 promotes an adaptive escape from MAPK pathway inhibition

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ABSTRACT

Oncogenic KRAS engages multiple effector pathways including the MAPK cascade to promote proliferation and survival of pancreatic cancer cells. KRAS-transformed cancer cells exhibit oncogene addiction to sustained activity of RAS for maintenance of malignant phenotypes. Previously, we have shown an essential role for the RHO guanine exchange factor ARHGEF2 for growth and survival of RAS-transformed pancreatic tumors. Here, we have determined that pancreatic cancer cells demonstrating KRAS addiction are significantly dependent on expression of *ARHGEF2*. Furthermore, enforced expression of ARHGEF2 desensitizes cells to pharmacological MEK inhibition and initiates a positive feedback loop which activates ERK phosphorylation and the downstream *ARHGEF2* promoter. Therefore, targeting *ARHGEF2* expression may increase the efficacy of MAPK inhibitors for treatment of RAS-dependent pancreatic cancers.

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Introduction

The dependency on sustained flux through pro-proliferative signaling pathways leads to cellular vulnerabilities that present opportunity for therapeutic intervention. Constitutive activation of oncogenes such as RAS, can lead to cellular addiction to continued expression of the oncogene and its downstream activity.⁴⁷ Therefore, oncogenic signaling pathways are drug targets that can be exploited to halt mechanisms required for cancer cell growth and survival.

Small GTPases are molecular switches that cycle between active GTP-bound and inactive-GDP bound forms that regulate a variety of cellular signaling events including growth, cellular differentiation, cell motility and survival.^{12,23} Arguably the most common of the small GTPases, the Ras superfamily, include 5 main family members Ras, Rho, Ran, Rab, and Arf. These families can be further divided into subfamilies that share the common core G domain providing essential GTPase and nucleotide exchange activity to regulate discrete cellular processes.^{14,48}

RAS is the prototypical member of the Ras family and is encoded by 3 proto-oncogenes, KRAS, HRAS and NRAS, which are frequently found mutated in human cancers.¹³ Of these, KRAS mutations are most frequent,

and are present in 33% of all tumors, including 95% of pancreas, 61% of colon and 17% of lung cancers. KRAS activation is coupled to transcription through the activation of multiple effector pathways including RAF-MAPK, PI3K, RALGDS, TIAM1-RAC and ARHGEF2-RHOA that underlie the phenotypes associated with pancreatic and other cancers.^{9-11,15,31,37,41} Finding therapeutically tractable targets in RAS effector pathways is critical for effective treatment of RAS driven cancers.

Pancreatic cancer is a highly metastatic deadly solid malignancy with a 5 y survival rate of less than 5%. Activating mutations in KRAS are nearly ubiquitous in pancreatic ductal adenocarcinoma (PDAC) occurring in 90–95% of cases.^{1,24,42} Since mutation of KRAS is an important driver of PDAC often occurring with up regulation and hyperactivation of EGFR1/2, suggests pharmacological agents aimed at inhibition of the MAPK pathway offer promising treatments.^{2,7,25,46} However, combined treatment of MAPK inhibitors with current chemotherapy agents such as gemcitabine do not improve clinical outcome.^{18,30,45} The effectiveness of pharmacological agents that target KRAS effectors often results in activation of compensatory pathways that limit their efficacy

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highlighting the importance of identifying mechanisms of adaptive response to MAPK inhibitory agents.

The RHO guanine exchange factor ARHGEF2 (also known as GEF-H1) is a microtubule associated guanine exchange factor and Dbl family member demonstrating exchange activity toward RHOA.³⁴ ARHGEF2 was found to contribute to cell survival and growth in KRAS and HRAS-transformed cells.⁹ Furthermore, the oncogenic potential of ARHGEF2 has been demonstrated in NIH-3T3 fibroblast transformation assays in nude mice.³ ARHGEF2 plays a critical role in supporting RAS transformation as depletion of *ARHGEF2* hinders the growth of pancreatic xenografts *in vivo*.⁹ We have found that *ARHGEF2* is a transcriptional target of oncogenic KRAS and the *ARHGEF2* promoter is transactivated downstream of multiple signaling pathways including MAPK and PI3K.²⁰

It is possible that RAS activation of the MAPK pathway is coupled to microtubule function through ARHGEF2 to promote and coordinate mitogenic signals with changes in cell shape, migration and morphogenesis. Indeed, cellular perturbation by mechanical strain has been shown to activate ERK in a RHOA dependent manner.^{22,39} Previously, we have demonstrated that ARHGEF2 contains negative regulatory sequences between amino acids 87–151 which sequester ARHGEF2 to the microtubules.²⁶ Lacking the microtubule binding domain, ARHGEF2^{Δ87–151} interacts with KSR-1, the best characterized scaffold for the MAPK pathway and was sufficient for activation of MEK1/2 and ERK1/2 phosphorylation in the absence of either PDGF or oncogenic RAS.⁹

Expression of ARHGEF2 is correlated with KRAS dependency and growth of pancreatic cancer cell lines

Previous studies have described a spectrum of KRAS dependency for a panel of KRAS mutant cancer cell lines.⁴⁰ Since RNAi knockdown of *KRAS* in some cell lines activates apoptosis, the strict operational definition of “KRAS addiction” required that *KRAS* knockdown induced caspase-3 cleavage. This definition established a RAS dependency index (DI) for multiple lung and pancreatic cancer cell lines harboring *KRAS* mutations.

Using the validated shRNAs for *KRAS* described previously,⁴⁰ we subjected a panel of PDAC cell lines to shRNA mediated *KRAS* knockdown to establish a RAS DI for commonly used PDAC cell lines (Fig. 1A). Most of the cell lines tested demonstrated exquisite sensitivity to *KRAS* ablation with the exception of KP4 which was relatively resistant to shRNA mediated *KRAS* depletion. These results demonstrate multiple cell lines are dependent on the sustained expression of *KRAS* for growth and viability.

Since ARHGEF2 is required for pancreatic cancer cell growth and survival, we queried if the panel of PDAC cell lines were dependent on sustained *ARHGEF2* expression. Using previously validated shRNAs for *ARHGEF2*,⁹ we subjected the same panel of PDAC cell lines described above to shRNA mediated *ARHGEF2* knockdown to establish dependency of cell lines on *ARHGEF2* expression for growth (Fig. 1B). Similar to *KRAS* knockdown, most of the cell lines tested demonstrated striking sensitivity to ARHGEF2 ablation with the exception of KP4 which was relatively insensitive to either shRNA targeting *ARHGEF2*. As we anticipated, these results mirrored the sensitivity to *KRAS* knockdown. When LOG2 transformed DI for *ARHGEF2* was plotted versus the DI for *KRAS* we observed a linear relationship with an R2 Pearson correlation of 0.99 (Fig. 1C; $P = 2.0 \times 10^{-4}$). In addition, we also observed a significant Pearson correlation of 0.85 between *ARHGEF2* mRNA expression and the LOG2 ARHGEF2 DI in the panel of cell lines (Fig. 1C inset; $p = 0.01$). Collectively, these results show that PDAC cell lines which are *KRAS* dependent are also *ARHGEF2* dependent.

Consistent with previous results, when either Panc-1 or MiaPaCa-2 cells were treated with siRNA targeting *KRAS* a significant decrease in ARHGEF2 expression was observed (Fig. 1D). The reduced ARHGEF2 expression observed with *KRAS* knockdown was of similar level as that seen by targeting *ARHGEF2* with siRNA directly (Fig. 1D). We hypothesized that *ARHGEF2* knockdown would phenocopy *KRAS* knockdown in activation of apoptosis in PDAC cells with high DI. Therefore, we assessed levels of caspase-3 cleavage in Panc-1 and MiaPaCa-2 cells, cell lines at the opposite spectrum of RAS-*ARHGEF2* DI treated with siRNA targeting either *KRAS* or *ARHGEF2*. Panc-1 cells were relatively *KRAS* and *ARHGEF2* independent by the DI prediction (Fig. 1C) and demonstrated a relatively low apoptotic response when treated with siRNA targeting either *KRAS* or *ARHGEF2* (Fig. 1D). In contrast, MiaPaCa-2 cells were the most *KRAS* and *ARHGEF2* dependent cell line by the DI prediction in the panel of cell lines tested (Fig. 1C), and demonstrated a high apoptotic response as measured by caspase-3 cleavage when either *KRAS* or *ARHGEF2* were knocked down (Fig. 1D). We also observed a reproducible reduction in sustained activity of the MAPK pathway in MiaPaCa-2 cells treated with siRNAs targeting either *KRAS* or *ARHGEF2* as measured by decreased ERK1/2 phosphorylation (Fig. 1D). These results highlight an essential role for ARHGEF2 cooperativity with oncogenic KRAS required for optimal activation of the MAPK signaling pathway.

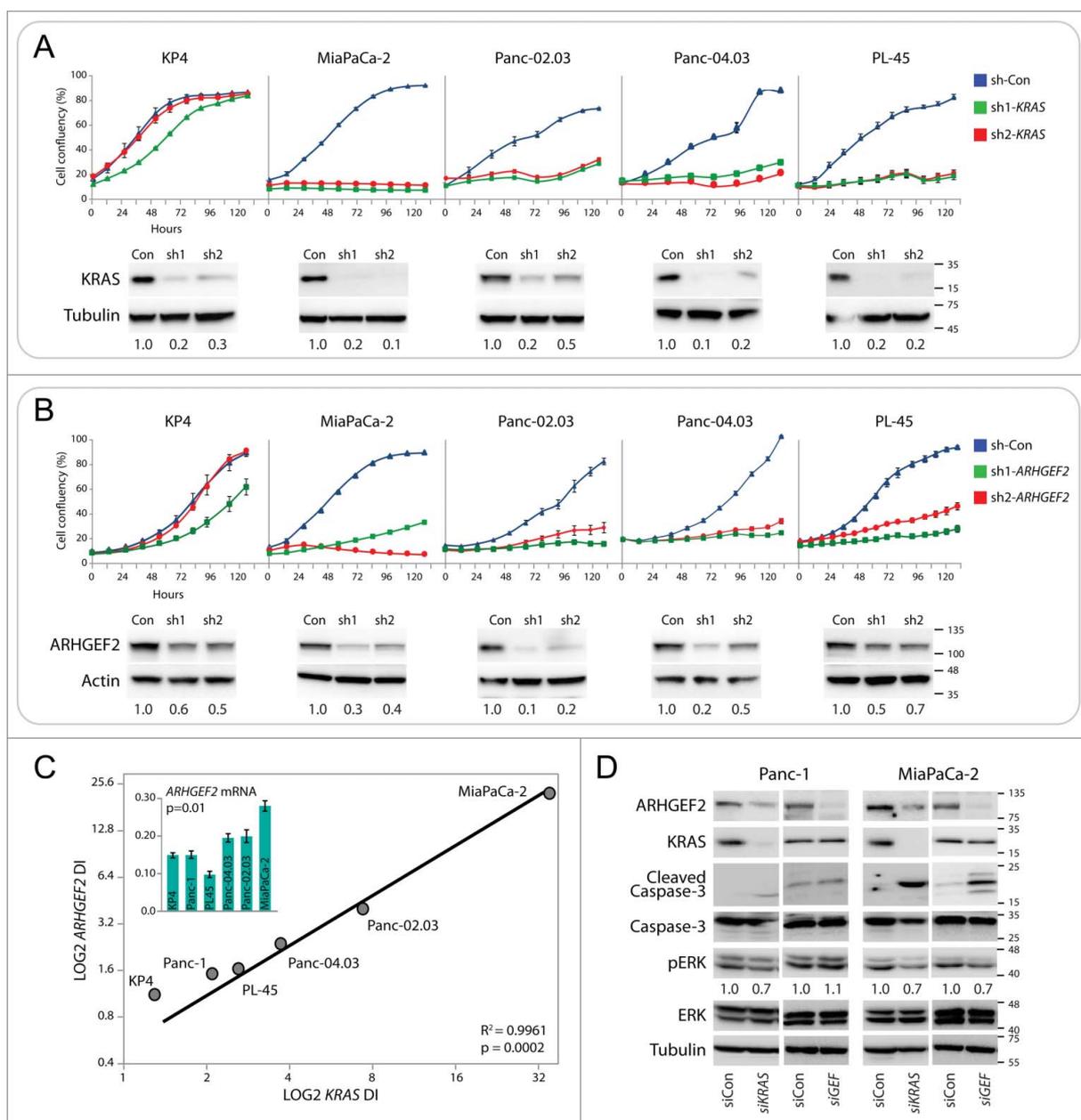


Figure 1. *ARHGEF2* expression correlates to *KRAS* dependency and influences survival. (A, B) Growth curves and western blots of the indicated cell lines expressing shRNA GFP control or one of 2 shRNAs targeting *KRAS* (A) or one of 2 shRNAs targeting *ARHGEF2* (B). Growth rates were monitored over the indicated time course using the Essen Incucyte Zoom. Western analysis of *KRAS* and *ARHGEF2* expression was examined 72 hours post infection. Quantification of *KRAS* (A) and *ARHGEF2* (B) is indicated. (C) Correlation between the LOG₂ normalized dependency index (DI) for *ARHGEF2* and *KRAS* in the indicated pancreatic cancer cell lines. Cells were treated with *KRAS* or *ARHGEF2* targeting shRNAs for 5 d and dependency index (DI) was calculated (see methods). Inset: β -actin normalized expression of *ARHGEF2* mRNA in the indicated pancreatic cancer cell lines. The p-value indicates significant Pearson correlation between *ARHGEF2* mRNA and LOG₂ *ARHGEF2* DI. (D) Western blot analysis of cleaved caspase-3 and p-ERK activation in Panc-1 and MiaPaCa-2 cell lines following acute knockdown of *KRAS* or *ARHGEF2* with siRNA (siRNA 5 nM final). Lysates were probed with indicated antibodies 72 hours post transfection. Quantification of p-ERK is the average from 4 independent measurements.

Activation of *ARHGEF2* promotes an adaptive escape pathway from MEK inhibition

Previous studies have shown that cancer cells can activate adaptive response pathways downstream of PI3K, Wnt or TGF- β to maintain cell survival and proliferation following

MEK1/2 inhibition.³⁶ We find these pathways also activate a minimal *ARHGEF2* promoter reporter construct²⁰ suggesting that up regulation of *ARHGEF2* could be an underlying mechanism for cellular escape from MEK inhibition. Therefore, we hypothesized that ectopic expression of *ARHGEF2* would desensitize cells to MEK1/2 inhibition.

Since active ARHGEF2^{Δ87-151} was shown to induce ERK1/2 phosphorylation,⁹ we challenged MiaPaCa-2 cells containing a doxycycline (dox) inducible ARHGEF2^{Δ87-151}-GFP to grow in the presence of multiple doses of the MEK1/2 inhibitor AZD6244. Currently in clinical trials, AZD6244 is a potent MEK1/2 inhibitor with IC₅₀ of 14 nM and blocks ERK1/2 activation in PDAC and colorectal cancer cell lines.⁴⁹ In the absence of dox, AZD6244 impaired MiaPaCa-2 cell proliferation with concomitant block in ERK1/2 phosphorylation (Fig. 2A and B; -dox). In contrast, induction of active ARHGEF2 expression by dox mitigated the effect of AZD6244 on MiaPaCa-2 cell proliferation even in the presence of high doses of drug (Fig. 2A; +dox). The enforced expression of ARHGEF2^{Δ87-151}-GFP potently activated ERK1/2 phosphorylation even at AZD6244 doses where MAPK signaling was inhibited in the absence of dox (Fig. 2B).

We previously demonstrated that expression of ARHGEF2 is activated through the MAPK pathway via a minimal RAS-responsive ARHGEF2 promoter

(AP-min). We envision that the AP-min construct can be used as a reporter for MAPK activity or signaling pathways that are able to activate expression of ARHGEF2. Using MiaPaCa-2 cells with dox inducible ARHGEF2^{Δ87-151}-GFP, we find luciferase expression from the AP-min construct was potently enhanced in cells treated with dox compared with empty vector control (Fig. 2C). Furthermore, dox induced ARHGEF2^{Δ87-151}-GFP can activate the AP-min promoter in the presence of AZD6244 (Fig. 2C). These results demonstrate that expression of ARHGEF2 establishes a positive feedback loop that activates the expression of ARHGEF2 through activation of the MAPK pathway and provides a mechanism for cellular escape from pharmacological MEK inhibition. We are currently conducting small molecule screens to uncover the determinants of ARHGEF2 promoter regulation that will in combination with existing treatment regimes, increase the efficacy of MEK1/2 inhibitors in clinical trials for treatment of PDAC and other RAS dependent cancers.

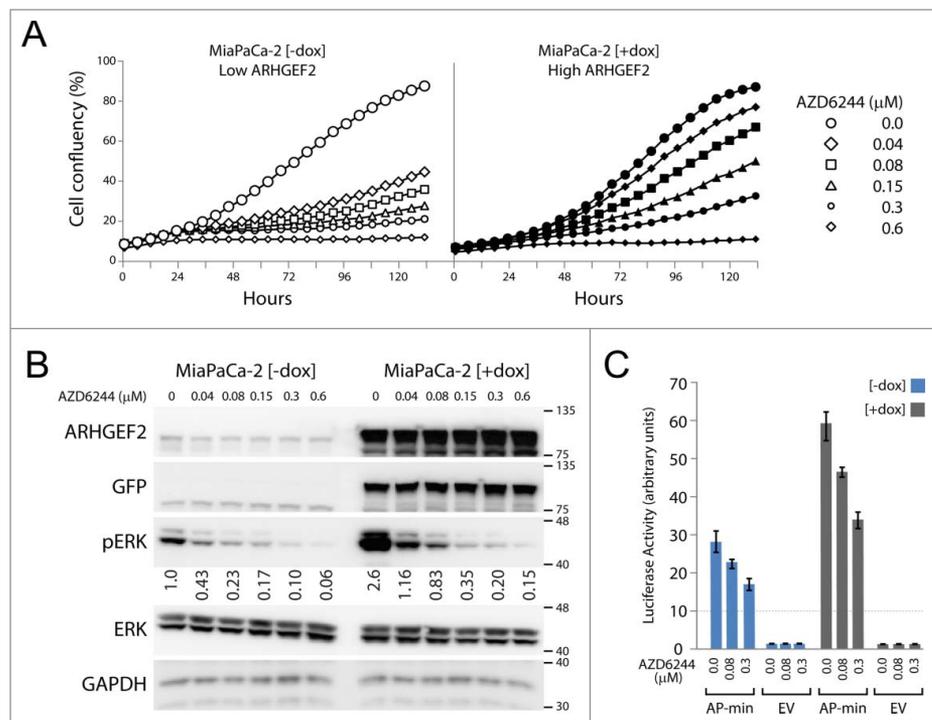


Figure 2. Enforced ARHGEF2 expression desensitizes cells to AZD6244 treatment via activation of the MAPK pathway. (A) Growth curves of doxycycline inducible ARHGEF2^{Δ87-151}-GFP-MiaPaCa-2 cells grown in the absence (open shapes, low ARHGEF2) or in the presence of doxycycline (filled shapes, high ARHGEF2) treated with the indicated doses of AZD6244. Growth rates were monitored over the indicated time course using the Essen Incucyte Zoom. (B) Western blot analysis of p-ERK activation in doxycycline inducible ARHGEF2^{Δ87-151}-GFP MiaPaCa-2 cells grown in the absence [-dox] or in the presence of doxycycline [+dox]. Lysates were probed with the indicated antibodies 24 hours post induction of ARHGEF2^{Δ87-151}-GFP. GAPDH served as a loading control. Quantification of p-ERK is indicated. (C) Normalized luciferase activity generated from the minimal ARHGEF2 promoter (AP-min) or pGL3-Basic empty vector control (EV) transfected in doxycycline inducible ARHGEF2^{Δ87-151}-GFP MiaPaCa-2 cells grown in the absence [-] or in the presence of doxycycline [+]. Cells were treated with the indicated doses of AZD6244 8 hours after induction of ARHGEF2. Luciferase activity was normalized to renilla expression and data are plotted as the fold change over empty vector. Error bars represent standard deviations from 3 independent transfections.

Discussion

The multistage process of cancer development is driven by the acquisition of genetic lesions that initiate and maintain cancer cell proliferation and survival.^{29,47} In some cases cancer cells can become dependent on or addicted to one or several oncogenes driving tumor initiation and/or tumor growth.¹⁶ The concept of “oncogene addiction” is also supported by preclinical and clinical data and suggests many cancers are sensitive to the inhibition of a single oncogene.²⁹ In the present study, we show that a panel of PDAC cell lines demonstrates a continuous distribution of KRAS dependency which correlates highly to ARHGEF2 dependency. These data suggest that one feature of KRAS dependency is coupled to the expression of *ARHGEF2*. Interestingly, circulating pancreatic tumor cells following surgery express a cell motility gene signature that includes increased levels of ARHGEF2 which was found to negatively predict overall survival.³⁸ Taken together, targeting *ARHGEF2* may be a novel therapeutic approach for treatment of RAS-dependent cancers.

Despite the high frequency of oncogenic RAS mutations in cancer,³² there are no clinically successful drugs that target RAS proteins directly. RAS has been viewed as a challenging target due to an apparent lack of drugable pockets.^{8,28} In fact, the NCI launched a ‘RAS Project’ in 2013 aimed at renewing efforts to find clinical inhibitors of RAS signaling. A large body of evidence suggests that RAS is a clinically relevant target since continued expression of mutant RAS is necessary for tumor initiation and maintenance. For example, RNAi knockdown of RAS has been shown to impair the *in vitro* and *in vivo* growth of PDAC cell lines.⁴ Similarly, in mouse models of PDAC driven by an inducible mutant RAS, lesions depended on continuous RAS expression for maintenance.^{6,50} However, after prolonged KRAS inactivation renewed tumor growth upon KRAS reactivation rapidly led to acquisition of resistance.⁶

RHO has a demonstrated role in the RAS-transformation program^{5,33,35} and can affect tumorigenic processes in multiple cancers.⁴³ Unlike RAS however, mutations of RHO in cancers are rare⁴⁴ suggesting RHO hyperactivation likely occurs through dysregulation of RHOGEFs and/or RHO GAPs downstream of oncogenes such as RAS. Although our results show that *ARHGEF2* is essential for the survival of PDAC cells likely through regulation of the MAPK pathway, whether the dependency on ARHGEF2 requires its RHOGEF activity remains to be determined. The enforced expression of ARHGEF2 could promote signal diversification to potentiate positive feedback through the MAPK pathway and increase RHOA-GTP signaling.²⁰ Whether these 2 events are

mechanistically linked is unclear. In HRAS transformed MEFs however, ERK1/2 phosphorylation by ARHGEF2 did not require its RHOGEF activity.⁹ In addition, previous studies have found that RHOA activation by oncogenic RAS may depend on cytosolic p190-RHO GAP activity⁵ rather than overexpression of a RHOGEF.

In the present study, we have used a constitutively active ARHGEF2 construct unable to associate with microtubules to uncouple localization and function. Microtubule repressed RHO signaling has been attributed to the microtubule sequestration of ARHGEF2 in an inactive state.²¹ Previously, we have found that pharmacological disruption of microtubules by nocodazole induces microtubule depolymerization and released ARHGEF2 from the microtubules resulting in a spatially defined activation of RHOA.²⁶ Furthermore, we have found that wild type MEFs treated with nocodazole potently activate ERK whereas *ARHGEF2*^{-/-} MEFs do not (Sandi and Rottapel, unpublished). These data establish a link between microtubule stability and the activation of RHO and RAS signaling pathways dependent on the cellular localization and therefore activation of ARHGEF2.

While RAS-effectors offer potential for therapeutic intervention, the efficacy of RAF and MEK inhibitors is primarily limited by the development of drug resistance. For example, the initial clinical response to Vemurafenib (a BRAF inhibitor) can be impressive in certain individuals but tumor resistance usually occurs after several months of treatment. Mechanisms of resistance include mutational activation of NRAS or receptor tyrosine kinase (RTK)-mediated activation of RAS, both leading to CRAF-dependent activation of MEK-ERK signaling.^{19,27} MEK has also become an attractive drug target and offers the promise of a novel therapeutic approach for RAS-mutant tumors.³⁶ AZD6244 is an allosteric MEK1/2 inhibitor that is highly selective, but has been found to exhibit modest clinical activity as a single agent and may show greater efficacy in combination therapies.³⁶ We have shown that enforced expression of active ARHGEF2 in Mia-PaCa-2 cells can activate ERK1/2 phosphorylation in the presence of relatively high doses of AZD6244. Interestingly, AZD6244 in combination with docetaxol, a microtubule stabilizing agent and hence deactivator of ARHGEF2²⁶ results in regression in human tumor xenograft models and is currently being investigated in clinical trials.¹⁷ Other reported mechanisms of MEK inhibition have been described including increased RAF dimerization, down regulation of the RAS GAP NF1, and up regulation of RTKs which promote increased flux through the ERK signaling pathway.³⁶

In summary, we have determined that PDAC cells demonstrating *KRAS* addiction are significantly dependent on expression of *ARHGEF2*. The results presented here and in our previous studies, show *ARHGEF2* signaling activates a positive feedback loop amplifying the MAPK pathway and is required for growth and survival of RAS-transformed cancer cells. Targeting *ARHGEF2* expression and signaling may increase the efficacy of MAPK pathway inhibitors and inhibitors of other RAS effectors for treatment of pancreatic cancer.

Methods

Cell culture

All cell lines used in the study (ATCC) were cultured as described previously.⁹ For siRNA mediated knockdown, cells (5.0×10^5) were transfected with siRNA against *KRAS* (siGenome) or *ARHGEF2* (Silencer select, Invitrogen) at 5 nM final concentration using RNAiMax (Invitrogen) according to the manufacturer's protocol. Knockdown was confirmed by western blot. Stable *ARHGEF2*^{Δ87–151}-GFP inducible cells were established as described previously.²⁰ To induce *ARHGEF2*^{Δ87–151}-GFP expression, cells were grown in media supplemented with 0.1 μg/mL doxycycline.

ARHGEF2 expression analysis

Cells were transfected with siRNA against *KRAS* (siGenome) or *ARHGEF2* (Silencer select, Invitrogen) at 1–5 nM final concentration using RNAiMax (Invitrogen) according to the manufacturer's protocol. Total RNA was isolated from cells with TRIZOL (Invitrogen). cDNAs were made using the QuantiTect kit (Qiagen). QPCR was performed using an ABI7900 system with the Fast-SYBR Green PCR core reagent (Applied Biosystems). Expression of *ARHGEF2* normalized to β-actin. Primer sequences as described previously.²⁰

Promoter assays

Luciferase reporter assays were conducted using the Dual-Luciferase Reporter Assay System (Promega) and reading on a Glo-Max dual injector luminometer (Promega) as described previously. The *ARHGEF2* promoter (AP-min) was defined as –264 to +23 relative to the TSS and cloned into pGL3-Basic (Invitrogen) as described previously.²⁰

Relative dependency index

Transient shRNA knockdown of *ARHGEF2* and *KRAS* was accomplished with lentivirus as described previously.⁹ Cell

lines were infected with hairpin control (shGFP) or *ARHGEF2* targeting shRNAs (sh*ARHGEF2*-1 and sh*ARHGEF2*-2) in parallel at an MOI of 5. After 48 hours of puromycin selection, infected cells were counted and replated at equal cell densities in 96 well plates in quadruplicate. Relative cellular viabilities were measured by quantitating optical densities (ODs) using AlamarBlue (ThermoFisher Scientific) when shGFP control-expressing cells reached confluence. ODs were used to calculate the relative dependency index using the formula: RDI at MOI of 5 = $(1/\text{OD shARHGEF2-1} + 1/\text{OD shARHGEF2-2}) \times \text{ODshGFP}$ (in each cell line tested). Proliferation rates were measured by plating 5000 cells/well in a 96-well plate and monitoring growth with Essen Incucyte Zoom (Essen Biosciences).

Statistical analysis

Statistical analysis was done using Student's t-test, assuming equal variance, and p-values were calculated based on 2-tailed test.

Western blot

Cells were lysed with 2X sample buffer and boiled to denature proteins. Standard protocols for western blots were followed and blots were imaged and quantified with BIO-RAD Quantity One. Antibodies used are as described previously.²⁰

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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