The RhoGEF GEF-H1 Is Required for Oncogenic RAS Signaling via KSR-1

Jane Cullis, 1,3,10 David Meiri, 1,10 Maria Jose Sandi, 1 Nikolina Radulovich, 1,5 Oliver A. Kent, 1 Mauricio Medrano, 1,3 Daphna Mokady, 1 Josee Normand, 1 Jose Larose, 1 Richard Marcotte, 1 Christopher B. Marshall, 1 Mitsuhioko Ikura, 1,3 Troy Ketela, 6,8 Jason Moffat, 5,8 Benjamin G. Neel, 1,3,5 Anne-Claude Gingras, 6,9 Ming-Sound Tsao, 1,3 and Robert Rottapel 1,2,3,4,7, * 1

Princess Margaret Cancer Center, University Health Network, 101 College Street, Room 8-703, Toronto Medical Discovery Tower, University of Toronto, ON M5G 1L7, Canada
2 Department of Medicine, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada
3 Department of Medical Biophysics, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada
4 Department of Immunology, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada
5 Department of Laboratory Medicine and Pathobiology, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada
6 Department of Molecular Genetics, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada
7 Division of Rheumatology, St. Michael’s Hospital, 30 Bond Street, Toronto, ON M5B 1W8, Canada
8 Donnelly Centre and Banting and Best Department of Medical Research, 160 College Street, Room 8-804, University of Toronto, Toronto, ON M5S 3E1, Canada
9 Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, 600 University Avenue, Room 992A, Toronto, ON M5G 1X5, Canada
10 These authors contributed equally to this work
* Correspondence: rottapel@uhnresearch.ca
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SUMMARY

Cellular transformation by oncogenic RAS engages the MAPK pathway under strict regulation by the scaffold protein KSR-1. Here, we report that the guanine nucleotide exchange factor GEF-H1 plays a critical role in a positive feedback loop for the RAS/MAPK pathway independent of its RhoGEF activity. GEF-H1 acts as an adaptor protein linking the PP2A B' subunits to KSR-1, thereby mediating the dephosphorylation of KSR-1 S392 and activation of MAPK signaling. GEF-H1 is important for the growth and survival of HRASV12-transformed cells and pancreatic tumor xenografts. GEF-H1 expression is induced by oncogenic RAS and is correlated with pancreatic neoplastic progression. Our results, therefore, identify GEF-H1 as an amplifier of MAPK signaling and provide mechanistic insight into the progression of RAS mutant tumors.

INTRODUCTION

The centrality of the RAS/MAPK pathway in promoting tumor formation is underscored by the high frequency of gain-of-function mutations in RAS family members and other components of the pathway in human cancers. KRAS has a particularly high mutation frequency of 30%–50% in colon and greater than 90% in pancreatic adenocarcinomas (Oliveira et al., 2007; Mulcahy et al., 1998). The importance of RAS is a reflection of its essential role in mediating the transduction of signals from growth factor receptors to pathways that regulate transcription, cell cycle progression, cell shape, and cell survival, all of which are commonly disturbed in cancer (Macara et al., 1996). RAS engages diverse signaling pathways, including RAF, PI3K, RAL-GDS, and TIAM-1, each of which are also subject to activating mutations in cancer (Davies et al., 2002; Samuels and Velculescu, 2004; Philp et al., 2001; Sjöblom et al., 2006; Greenman et al., 2007; Engers et al., 2000). RAS activation is coupled to transcription through the activation of the MAPK cascade, involving the sequential phosphorylation and activation of the serine/threonine kinases RAF (MAPKKK), MEK1/2 (MAPKK), and ERK1/2 (MAPK).

Significance

Mutational activation of RAS occurs in over 90% of pancreatic cancers and is required for both the initiation and progression of tumorigenesis. However, the mechanism of RAS-mediated cellular transformation is not fully understood. Here, we find that GEF-H1 is necessary for optimal RAS/MAPK pathway signaling and contributes to the growth and survival of RAS mutant cells. GEF-H1 expression is induced by oncogenic RAS and is elevated in pancreatic tumor samples, thereby providing an amplifying loop for RAS/MAPK signaling. Our results, therefore, extend our understanding of the signaling dependencies of oncogenic RAS, which may ultimately improve the development of RAS-pathway-directed therapeutics.
GEF-H1 is a catalytic (C), structural (A), and regulatory (B) subunit. The catalytic and structural subunits are constitutively associated to form a core complex to which one of many B subunits can bind (Janssens and Goris, 2001). Four different B subunits (B’, B”, and B””) exist in mammals that determine the localization and substrate specificity of the holoenzyme (Janssens and Goris, 2001). The A and C subunits constitutively associate with KSR-1, whereas association of the B” subunit is induced only upon RAS activation (Ory et al., 2003). The mechanism by which the B” subunit is recruited to KSR-1 has yet to be elucidated.

GEF-H1, which is encoded by ARHGEF2, is a microtubule-associated guanine nucleotide exchange factor (GEF) for the Rho family of small GTPases (Ren et al., 1998). Several lines of evidence have highlighted the transforming potential of GEF-H1. ARHGEF2 is amplified in hepatocellular carcinoma (Cheng et al., 2012) and is a transcriptional target of gain-of-function p53 mutants (Mizuarai et al., 2006) and the metastasis-associated hPTTG1 (Liao et al., 2012). Truncated versions of GEF-H1 can transform NIH 3T3 cells (Whitehead et al., 1995) and induce tumor formation in nude mice (Brecht et al., 2005). ARHGEF2 is one of six genes significantly downregulated in response to imatinib treatment in gastrointestinal tumors (Frolov et al., 2003). In addition, ARHGEF2 was identified in a genome-wide pooled small hairpin RNA (shRNA) screen designed to identify genes that are required for the survival of human breast, colon, lung, ovarian, and pancreatic cell lines (Mardco et al., 2012). These data suggest that GEF-H1 may be a marker for and/or contribute to tumorigenesis in multiple contexts.

RESULTS

GEF-H1 Contributes to the Survival of a Subset of Human Cancer Cell Lines, and Its Expression Is Regulated by the RAS/MAPK Pathway

GEF-H1 was found to contribute to the competitive growth characteristics of 18 out of 73 cell lines, 13 of which were identified in the original shRNA screen and 5 of which were identified in our secondary screen (Figure 1A and Table S1 available online). For further validation, we selected three of these cell lines and stably infected them with two distinct lentiviral hairpins directed against GEF-H1. Cells depleted of GEF-H1 exhibited decreased growth and increased death relative to control hairpin-expressing cells as assessed by caspase 3 cleavage (Figures 1B and S1A–S1H). These data suggest that GEF-H1 is important for cell growth and survival in several human cell lines derived from different tumor types.

We noted that GEF-H1 dependency was enriched in RAS/BRAF mutant cell lines (13 of 30 [43.3%]) compared to RAS/BRAF wild-type cell lines (5 of 43 [11.6%]). GEF-H1 was found to contribute to cell growth/survival in 10 of 25 (40%) KRAS mutant pancreatic cancer cell lines with little effect on the three wild-type KRAS pancreatic cancer cell lines (Table S1). We therefore explored the possibility that GEF-H1 sensitivity in some cellular contexts is epistatic with gain-of-function mutations in the RAS/MAPK pathway. Because elevated expression of GEF-H1 is transforming in NIH 3T3 cells (Whitehead et al., 1995), we examined the ability of mutant RAS family members to induce GEF-H1 expression in a common isogenic cellular background. We observed that GEF-H1 protein levels were increased in cells transformed by each mutant RAS family member compared to nontransformed cells (Figure 1C). We next determined whether the induction of GEF-H1 expression was a direct result of activated RAS or a secondary consequence of the transformed state. We used a murine embryonic fibroblast (MEF) cell line expressing a hydroxytamoxifen (4-OHT)-inducible form of HRAS V12 (ER:HRASV12) and found GEF-H1 was found to contribute to cell growth/survival in 10 of 25 (40%) KRAS mutant pancreatic cancer cell lines with little effect on the three wild-type KRAS pancreatic cancer cell lines (Table S1). We therefore explored the possibility that GEF-H1 sensitivity in some cellular contexts is epistatic with gain-of-function mutations in the RAS/MAPK pathway. Because elevated expression of GEF-H1 is transforming in NIH 3T3 cells (Whitehead et al., 1995), we examined the ability of mutant RAS family members to induce GEF-H1 expression in a common isogenic cellular background. We observed that GEF-H1 protein levels were increased in cells transformed by each mutant RAS family member compared to nontransformed cells (Figure 1C). We next determined whether the induction of GEF-H1 expression was a direct result of activated RAS or a secondary consequence of the transformed state. We used a murine embryonic fibroblast (MEF) cell line expressing a hydroxytamoxifen (4-OHT)-inducible form of HRAS V12 (ER:HRASV12) (Gupta et al., 2007) and found that GEF-H1 expression increased within 15 min of ER:HRASV12 induction and continued to increase with progressive elevation of ER:HRASV12 expression (Figure 1D, upper panel). Cells treated with vehicle control (EtOH) exhibited no change in GEF-H1 levels (Figure 1D, lower panel). These data show that GEF-H1 is induced acutely in response to expression of HRAS V12. MAP kinase pathway activation followed a bimodal distribution, peaking at 15 min and 8 hr after HRAS V12 induction, but decreasing over intermediate time points, as has been previously shown (Gupta et al., 2007).

ARHGEF2 Is a Transcriptional Target of the RAS/MAPK Pathway

To assess whether GEF-H1 expression was dependent on MAPK activation, we treated OV-90, HCT116, and Panc 02.03 cells with the MEK1/2 inhibitors PD98059 and U0126 and found that the GEF-H1 protein level decreased following MEK1/2 inhibition (Figure 2A). Similar findings were observed in HRAS V12-transformed NIH 3T3 cells (Figure 2B). We noted that the GEF-H1 mRNA level was elevated 2-fold in HRAS V12-transformed NIH 3T3 cells relative to wild-type cells (Figure 2C) and sought to determine whether ARHGEF2 was a direct...
transcriptional target of mutant RAS. Based on phylogenetic footprinting and CpG island enrichment, we identified a 1.9 kb region upstream of the first exon of murine Arhgef2 predicted to contain the putative promoter, and we cloned this region into a luciferase reporter (Figure 2D). Expression of HRASV12-induced a 7-fold increase in the normalized Arhgef2 promoter-mediated luciferase activity compared to NIH 3T3 cells expressing the Arhgef2 promoter alone and was quenched following MEK inhibition (Figure 2E). Together, these data show that Arhgef2 is a transcriptional target of the RAS/MAPK pathway and that the elevated GEF-H1 protein level observed in HRASV12-transformed cells are, at least in part, due to elevated transcription.

To address the role of GEF-H1 in supporting tumor formation of HRASV12-transformed NIH 3T3 cells, we generated subcutaneous tumor xenografts in NCr nude mice. Parental and shGFP-expressing cells formed tumors within 10 days of injection, while GEF-H1-depleted cells demonstrated attenuated tumor growth (Figures 3D, 3E and S2B). Moreover, GEF-H1-depleted tumors exhibited increased caspase 3 cleavage (Figure 3F). To further examine the role of GEF-H1 in HRASV12-mediated cell survival, we monitored the behavior of MEFs derived from Arhgef2 knockout mice (Arhgef2−/−) following ectopic expression of HRASV12 (Figure 3G). Extensive cell death was observed in Arhgef2−/− compared to wild-type MEFs following HRASV12.
expression. Re-expression of GEF-H1 in Arhgef2−/− MEFs expressing HRASV12 restored cell viability. These data show that GEF-H1 contributes to HRASV12-mediated cell transformation and cell viability in vitro and in vivo.

**GEF-H1 Induction and Dependency in BRAFV600E-Expressing Cell Lines**

Because OV-90 carries the activating BRAFV600E mutation (Estep et al., 2007), we queried whether BRAFV600E similarly induced GEF-H1 protein expression. We found that GEF-H1 protein levels were increased in BRAFV600E-transformed NIH 3T3 cells and were sensitive to MEK inhibition (Figures S2C and S2D). Moreover, GEF-H1 expression in the human melanoma cell line A375, which carries an endogenous BRAFV600E mutation, was suppressed following MEK inhibition (Figure S2E). BRAFV600E expression also induced a 4.6-fold increase in the normalized Arhgef2 promoter-mediated reporter expression compared to NIH 3T3 cells expressing the Arhgef2 promoter alone, which was suppressed with MEK inhibition (Figure S2F). Lastly, knockdown of GEF-H1 induced cell death in BRAFV600E-transformed cells (Figures S2G and S2H).

These data indicate that the induction of GEF-H1 expression and GEF-H1-dependent cell survival extends to other oncoproteins that activate the MAPK pathway.

**GEF-H1 Is Necessary for Optimal MAPK Pathway Activation in Response to HRASV12**

We next sought to investigate the mechanism underlying the contribution of GEF-H1 to HRASV12-mediated cellular transformation. We compared the levels of Rho-GTP in control and GEF-H1 knockdown cells expressing HRASV12 but found no change in Rho-GTP levels (Figure S3A and S3B), demonstrating that a change in Rho-GTP cannot account for the contribution of GEF-H1 in HRASV12-mediated transformation. We therefore investigated whether elevated levels of GEF-H1 affected the signaling characteristics of upstream components of the RAS/MAPK pathway as part of a potential positive feedback mechanism. We expressed HRASV12 in MEFs harboring stable knockdown of GEF-H1 and probed lysates for phosphorylated forms of MEK1/2 and ERK1/2 to assess MAPK pathway activity. MEK1/2 and ERK1/2 were highly phosphorylated in HRASV12-expressing MEFs (Figure 4A, lane 2), but, surprisingly, MEK1/2 and ERK1/2 phosphorylation was significantly reduced in GEF-H1-depleted cells (Figure 4A, lanes 4 and 6). Expression of an shRNA-resistant GEF-H1 (rGEF-H1) restored MEK1/2 and ERK1/2 phosphorylation in response to HRASV12 expression in GEF-H1 knockdown MEFs (Figure 4A, lane 7). A similar defect in HRASV12-mediated ERK1/2 phosphorylation was seen in
Figure 3. GEF-H1 Contributes to Cell Survival and Growth in HRASV12-Transformed Cells

(A) GEF-H1 protein levels in NIH 3T3 cells expressing vector, HRASV12, or HRASV12 with a control hairpin (shGFP) or two distinct murine GEF-H1 shRNAs (shGEFm1 and shGEFm2).

(B) Cells described in (A) were probed for caspase 3 cleavage by western blot 5 days after infection with lentiviral hairpins. Tubulin served as a protein loading control.

(C) Representative images of cell lines described in (A) grown for 10 days in 0.3% agar to form colonies. Scale bars, 200 μm.

(D) Photographs of NCr nude mice 14 days after subcutaneous injection of cells described in (A).

(E) Final mean tumor volumes are shown in (D). Results are the combination of four independent experiments (n = 21 tumors). Error bars indicate ± SEM.

(F) Immunohistochemistry of NIH 3T3-HRASV12 tumor sections stained for cleaved caspase 3. Four tumors were sampled from two independent experiments. Scale bars, 100 μm.

(G) Bright field images of wild-type or Arhgef2−/− MEFs expressing eGFP, eGFP-HRASV12, or eGFP-HRASV12 and Flag-GEF-H1 4 days after transfection and selection. Scale bars, 100 μm.

See also Figure S2.
Figure 4. GEF-H1 Is Necessary for Optimal MAPK Pathway Activation in Response to HRAS^{V12}

(A) MEFs stably expressing shGFP, shGEFm1, or shGEFm2 were transfected with empty vector or HRAS^{V12} and probed for pERK or pMEK by western blot. shGEFm2-expressing cells were co-transfected with HRAS^{V12} and Flag-rGEF-H1, Flag-rGEF-H1^{E243K}, or Flag-AKAPLbc. Expression of plasmids was confirmed by immunoblotting with anti-GEF-H1, anti-RAS, or anti-Flag (AKAPLbc) antibodies.

(B) Real-time NMR measurement of RhoA nucleotide exchange rates in lysates from HEK293T cells expressing eGFP, eGFP-GEF-H1, eGFP-GEF-H1^{D87–151}, eGFP-GEF-H1^{E243K}, or eGFP-p115RhoGEF. Graphical representation of eGFP-p115RhoGEF-induced nucleotide exchange rate is not to scale as indicated by breaks in graph, because the rate was 9.4-fold over eGFP-GEF-H1 (r = 0.132 versus r = 0.014). Data are representative of three independent experiments ± SD.

(C) Schematic representation of KSR-1 constructs used in (D).

(D) Pyo-tagged KSR-1 constructs were coexpressed with Flag-GEF-H1 in HEK293T cells. Protein complexes were immunoprecipitated with anti-Flag antibody, and proteins were detected by immunoblotting with anti-KSR-1 or anti-Flag antibodies.

(E) MEFs were transfected with vector or eGFP-GEF-H1^{D87–151}, and Ksr1^{+/−}/C0 MEFs were transfected with vector, eGFP-GEF-H1^{A87−151}, eGFP-GEF-H1^{A87−151} and Pyo-KSR-1, or Pyo-KSR-1 alone and assayed for pERK by western blot. GEF-H1 and KSR-1 expression was determined by western blot.

See also Figure S3.
Arhgef2−/− MEFs, which was restored by GEF-H1 expression (Figure S3C, lanes 4 and 5).

To determine the specificity of GEF-H1-dependent MAPK pathway activation, we attempted to rescue the GEF-H1 knockdown phenotype by expressing either AKAP-Lbc, the closest GEF family member to GEF-H1, or p115 RhoGEF, another Rho-GEF family member. Neither AKAP-Lbc (Figure 4A, lane 9) nor p115 RhoGEF (Figure S3C, lane 7) rescued MEK1/2 and ERK1/2 phosphorylation in response to acute HRASV12 expression in GEF-H1 knockdown or Arhgef2−/− MEFs, respectively, despite 9-fold greater catalytic activity of p115RhoGEF compared to GEF-H1 (Figure 4B). To investigate whether GEF-H1-mediated MAPK pathway activation was dependent on its GEF activity, we coexpressed a catalytically inactive, shRNA-resistant form of GEF-H1 (rGEF-H1E243K, Figure 4B) with HRASV12 in MEFs we coexpressed a catalytically inactive, shRNA-resistant form of GEF-H1 (rGEF-H1E243K, Figure 4B) with HRASV12 in MEFs. We found that MEK1/2 and ERK1/2 phosphorylation was dependent on its GEF activity, with only 3.5% of cells (3 of 87) underwent PDGF-dependent membrane translocation in Arhgef2−/− cells (Figure 5A, columns 3 and 4; Figure S4A), a defect that was rescued by the expression of wild-type GEF-H1 (Figure 5A, columns 5 and 6; Figure S4A), with 30% (21 of 71) of cells expressing only KSR-1S392A restored cellular viability (Figures 5D and 5E). These data suggest that the growth factor dependence of KSR-1 translocation to the plasma membrane is contingent on the release of GEF-H1 from the microtubule array. Importantly, we found that the endogenous interaction of GEF-H1 and KSR-1 was induced between 5–20 min of PDGF stimulation, correlating with their translocation to the plasma membrane (Figure 5C). These data suggest that endogenous complex formation between GEF-H1 and KSR-1 occurs on the plasma membrane in response to PDGF treatment.

To clarify whether the dependence of HRASV12 on GEF-H1 for cell survival was mediated through KSR-1, we measured cell viability following ectopic expression of wild-type KSR-1 or KSR-1S392A with HRASV12 in Arhgef2−/− MEFs and found that only KSR-1S392A restored cellular viability (Figures 5D and 5E). These data provide genetic evidence that dephosphorylation of the negative regulatory site S392 on KSR-1 is the critical target downstream of GEF-H1 that supports cell survival in HRASV12-transformed cells.

To determine whether GEF-H1 regulation of the HRASV12/MAPK cascade is coupled to the dephosphorylation of KSR-1, we asked whether wild-type KSR-1 or KSR-1S392A could restore HRASV12-induced ERK1/2 phosphorylation in the absence of GEF-H1, HRASV12 expression induced ERK1/2 phosphorylation in control hairpin-expressing MEFs, but not in cells depleted of GEF-H1 (Figure 5F, lanes 2 and 4). High expression of rGEF-H1 in GEF-H1-depleted cells greatly enhanced ERK1/2 activation in response to HRASV12, supporting the model that increased levels of GEF-H1 result in amplification of the MAPK pathway.  

GEF-H1 is a Component of the KSR-1 Complex and is Required for the Dephosphorylation of the Negative Regulatory Site of KSR-1

Given that GEF-H1 catalytic activity is dispensable for HRASV12-dependent MAPK pathway activation, we hypothesized that GEF-H1 may be providing a scaffold function for components of the MAPK pathway. We investigated whether GEF-H1 could form a complex with KSR-1, the major scaffold for the MAPK pathway. We detected an interaction between endogenous GEF-H1 and endogenous KSR-1 in GEF-H1 immunoprecipitates from wild-type, but not Arhgef2−/− MEFs (Figure S3D). Similarly, in an overexpression system, we detected an interaction between KSR-1 and a mutant of GEF-H1 that deletes an RhoGEF family member to GEF-H1, or p115 RhoGEF, another Rho-GEF family member. Neither AKAP-Lbc (Figure 4A, lane 9) nor p115 RhoGEF (Figure S3C, lane 7) rescued MEK1/2 and ERK1/2 phosphorylation was fully restored (Figure 4A, lane 8). These findings were confirmed in Arhgef2−/− MEFs (Figure S3C, lane 6). These data show that GEF-H1 potentiates the HRASV12/MAPK pathway in a manner independent of its GEF activity.

Importantly, we found that the endogenous interaction of GEF-H1 and KSR-1 was induced between 5–20 min of PDGF stimulation, correlating with their translocation to the plasma membrane (Figure 5C). These data suggest that endogenous complex formation between GEF-H1 and KSR-1 occurs on the plasma membrane in response to PDGF treatment.

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Cancer Cell

GEF-H1 Is Required for Oncogenic RAS Signaling

A

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B

KSR-1 +eGFP
KSR-1-E393QA +eGFP
KSR-1 +eGFP-GEF-H1 +eGFP-GEF-H1

C

IP: GEF-H1 (endogenous)
BD: GEF-H1 (endogenous)

D

Arhgef2+/+ +eGFP-HRAS123 +eGFP-HRAS123 +eGFP-HRAS123 KSR-1-E393QA KSR-1

F

shGFP shGEFm2

E

Cell number

Time (hours)

- HRAS123
- GEF-H1
- KSR-1
- pERK
- RAS
- ERK

(legend on next page)
cascade (Figure 5F, lane 5). Expression of KSR-1T392A efficiently restored HRASV12-mediated ERK1/2 phosphorylation in GEF-H1 knockdown cells compared to wild-type KSR-1 (Figure 5F, lanes 6 and 7). These data demonstrate that dephosphorylation of KSR-1 T392 is sufficient to overcome the GEF-H1-dependence of HRASV12-mediated ERK1/2 activation.

**GEF-H1 Is Required for PP2A-Mediated Dephosphorylation of KSR-1**

In an independent study, we identified GEF-H1 as a PP2A interacting partner in a proteomic screen designed to probe for proteins that bound to the PP2A catalytic subunit (D.M., C.B.M., J.L., M. Mullin, A.-C.G., M.I., and R.R., unpublished data) and found that GEF-H1 interacts with the B’ regulatory PP2A subunits (PPP2R5A, PPP2R5B, and PPP2R5E). We hypothesized that GEF-H1 may function as a bridge between KSR-1 and PP2A to control KSR-1 S392 dephosphorylation. First, we confirmed the previously published data showing an interaction between KSR-1 and the B’ regulatory PP2A subunits (Figure 6A) (Ory et al., 2003). We observed that GEF-H1 bound to the same PP2A subunits that interacted with KSR-1 (Figure 6A). We next determined the regions of GEF-H1 involved in PP2A and KSR-1 binding by expressing deletion mutants of GEF-H1 (Figure 6B) and probing for the catalytic subunit of PP2A and KSR-1 in GEF-H1 immune complexes (Figure 6C). Analysis of GEF-H1 immunoprecipitates revealed that endogenous KSR-1 interacted with full-length GEF-H1, GEF-H1(236-572), and GEF-H1(236-433). These results localize the binding site for KSR-1 to the DH domain of GEF-H1, while endogenous PP2Ac binds to the GEF-H1 PH domain (Figure 6C). These data show that KSR-1 and PP2A bind to distinct sites on GEF-H1 and suggest that GEF-H1 may function to bridge PP2A to KSR-1.

To determine whether GEF-H1 acts as a bridge to link KSR-1 to PP2A, we stably infected human embryonic kidney 293T (HEK293T) cells expressing the PP2A B’ subunit with an shRNA targeting GEF-H1 and probed PP2A immunoprecipitates for endogenous KSR-1 (Figure 6D). KSR-1 was detected in immune complexes of PP2A B’ subunits in shGFP-expressing cells, but not those depleted of GEF-H1. Thus, the interaction between KSR-1 and PP2A is dependent on GEF-H1. These data support a model whereby GEF-H1 provides a bridging function to recruit the PP2A B’ subunits required for the dephosphorylation of the negative regulatory S392 site on KSR-1 and activation of the MAPK pathway.

Given that dephosphorylation of KSR-1 on S392 is induced in response to PDGF and oncogenic RAS, we sought to determine whether the interaction between GEF-H1 and KSR-1 was similarly regulated. We isolated Flag-PPP2R5E immune complexes from HEK293T cells and probed them for the presence of GEF-H1 and KSR-1 after PDGF treatment (Figure 6E). Although GEF-H1 and KSR-1 were not detectable in PPP2R5E immune complexes in starved cells, both GEF-H1 and KSR-1 were recruited to PPP2R5E immune complexes after 5 min of PDGF stimulation and disappeared after 15 min (Figure 6E). Moreover, induction of oncogenic HRAS with 4-OHT induced a protein complex composed of endogenous KSR-1, GEF-H1, and PPP2R5E proteins after 20 min and extending to 90 min following RAS activation (Figure S5). These data show that PDGF or HRASV12 induce the formation of a KSR-1, GEF-H1, and PP2A protein complex. Moreover, the complex appears to be temporally regulated, suggesting the presence of feedback mechanisms that attenuate its assembly even with constitutive activation of the pathway.

**GEF-H1 Is Important for the Growth of RAS Mutant Pancreatic Tumor Xenografts**

Over 90% of human pancreatic ductal adenocarcinomas (PDACs) harbor activating mutations in KRAS (Smit et al., 1988). We evaluated whether GEF-H1 expression was increased in PDAC by immunohistochemistry on pancreatic tissue microarrays (TMAs). We probed tissue sections of 14 normal pancreatic ducts, 32 PanIN-1 (A and B) lesions, 9 PanIN-2 and IN-3 lesions, and 14 PDAC tumor samples for GEF-H1 expression using a monoclonal antibody against GEF-H1 (Figure 7A). Normal pancreatic ducts and PanIN-1 lesions did not express GEF-H1, whereas greater than 90% (21 out of 23) of the more advanced histologic grades expressed GEF-H1 (Figures 7A and S6A). These data demonstrate that GEF-H1 expression is positively correlated with neoplastic progression of pancreatic tumors. Treatment of the PDAC cell line PAC-1, harboring a KRASV12 mutation, with MEK inhibitors PD98059 or UO126 resulted in reduced GEF-H1 levels (Figure S6B). Together, these data show that GEF-H1 expression is increased in PDAC cells in a manner that is dependent on MAPK pathway activation.

To determine whether GEF-H1 was necessary for MAPK pathway activation in PDAC cells, we knocked down GEF-H1 in PanC-1 cells and observed increased KSR-1 S392 phosphorylation and a corresponding decrease in ERK1/2.
Figure 6. GEF-H1 Is Required for PP2A-Mediated Dephosphorylation of KSR-1 on S392

(A) Flag-PP2A immune complexes were isolated from stable Flag-PP2A catalytic and regulatory subunit-expressing HEK293T cells using anti-Flag antibodies. Flag-PP2A complexes were probed for endogenous GEF-H1 and endogenous KSR-1 (rows 2 and 3). Total expression levels of GEF-H1 and KSR-1 in lysates are shown in rows 4 and 5.

(B) Schematic representation of GEF-H1 constructs used in (C).

(C) Flag-tagged truncated variants of GEF-H1 were expressed in HEK293T cells, and protein complexes were immunoprecipitated with anti-Flag antibodies. Lysates were probed with anti-KSR-1 or anti-PP2Ac antibodies.

(D) HEK293T cells stably expressing Flag-tagged PP2A regulatory subunits were infected with shGFP or shGEFh2 lentiviruses. Flag-PP2A subunits were immunopurified with anti-Flag (row 1) and probed for endogenous KSR-1 (row 2). Flag-PP2A subunit expression (row 3) and GEF-H1 knockdown (row 4) were confirmed by immunoblotting lysates with Flag and GEF-H1 antibodies, respectively.

(E) HEK293T cells stably expressing Flag-PPP2R5E were treated with 25 ng/ml PDGF for 0, 5, 10, or 15 min. Flag-PPP2R5E immune complexes were probed for the presence of endogenous GEF-H1 and KSR-1 (left panel). Lysates were probed for total levels of Flag-PPP2R5E, endogenous GEF-H1 and KSR-1 (right panel).

See also Figure S5.
phosphorylation compared with control hairpin-expressing cells (Figure 7B). Expression of shRNA-resistant GEF-H1^{A87–151} restored the basal levels of phosphorylated KSR-1 and ERK1/2 in GEF-H1-depleted cells (Figure 7B). These data indicate that GEF-H1 is both necessary and sufficient for KSR-1 S392 dephosphorylation and ERK1/2 activation in PDAC cells harboring endogenous RAS mutations. Expression of KSR-1^{S392A}, but not wild-type KSR-1, corrected the defect in the phosphorylated ERK levels in GEF-H1 knockdown cells, showing that active KSR-1 can circumvent the need for GEF-H1 in PANC-1 cells (Figure 7B).

We tested the contribution of GEF-H1 to the in vitro cell growth of four human KRAS mutant pancreatic cancer derived cell lines including PANC-1 Panc 08.13, Panc 04.03, and PL-45 (Figures S6C–S6F). These cell lines displayed varying sensitivity to GEF-H1 depletion for cell growth (a 50%, 90%, 80%, and 65% reduction, respectively) compared to control hairpin-expressing cells. Of the six pancreatic lines that we had tested, we examined the contribution of GEF-H1 to tumor growth in three of these lines, PANC-1 (KRAS^{G12}), HPAF-II (KRAS^{G12}), and BxPC3 (wild-type KRAS), in immune-deficient mice. PANC-1 and HPAF-II cells exhibited profound attenuation of tumor growth relative to control hairpin cells (Figures 7C, S6G, and S6H). The tumor growth of BxPC3 cells was not affected by depletion of GEF-H1, highlighting the dependency of oncogenic RAS on GEF-H1 (Figures 7C and 56B). In addition, increased tumor-associated caspase 3 cleavage was observed in PANC-1 xenografts (Figures S6J). Collectively, our data demonstrate an amplifying feedback loop involving GEF-H1 in the RAS/MAPK pathway across a variety of cell types expressing different mutant RAS family members. These data support the model that GEF-H1 is important for the growth of tumor cells harboring activating mutations in RAS.

**DISCUSSION**

Signaling through the RAS/MAPK pathway is gated by KSR-1, a highly conserved scaffold protein that ensures strict spatio-temporal regulation of ERK activation. Genetic studies have demonstrated a critical requirement of KSR-1 for growth factor-mediated signaling through the RAS/MAPK pathway (Sieburth et al., 1999; Lozano et al., 2003) and the formation of HRAS^{V12}-dependent tumors (Xiao et al., 2010). The requirement of KSR-1 in HRAS^{V12}-mediated transformation is strictly dependent on the dephosphorylation of KSR-1 at S392 by PP2A (Razidlo et al., 2004). In this study, we provide a mechanistic explanation of how the B’ subunit is recruited to the PP2A/KSR-1 complex and uncover a positive feedback loop involving the RhoGEF GEF-H1 that is necessary for HRAS^{V12}-mediated transformation. We show that Arhgef2 is a direct transcriptional target of the RAS/MAPK pathway, and its elevated protein expression is similarly responsive to oncogenic BRAF and H-, K-, and NRAS family members. We demonstrate that GEF-H1 contributes to the growth and survival of BRAF^{V600E} and HRAS^{V12}-transformed NIH 3T3 cells and PDAC xenografts. We anticipate that there may be examples of escape mechanisms whereby some RAS mutant tumors no longer depend on the GEF-H1 amplifying loop, which will be an area of future investigation.

The discovery that a RhoGEF is involved in a positive feedback loop for the MAPK pathway suggests a model whereby amplification of the MAPK pathway could be coupled to signal diversification through the activation of RhoA, a known component of the RAS transformation program (Qiu et al., 1995; Prendergast et al., 1995; Sahai et al., 2001; Chen et al., 2003). Our data suggest that oncogenic RAS induces RhoA-GTP independently of GEF-H1, a finding consistent with the previously reported model that a decrease in p190RhoGAP activity, rather than an increase in total cellular RhoGEF activity, controls RhoA-GTP levels in HRAS^{V12}-transformed cells (Chen et al., 2003). The observation that overexpression of GEF-H1 is sufficient to increase MEK1/2 and ERK1/2 phosphorylation raises the possibility that the oncogenic potential of GEF-H1 is mediated through its capacity to increase cellular Rho-GTP levels and/or activate the MAPK pathway.

An important implication that follows from this study is the possibility that mitogenic signals conveyed through the MAPK pathway might be coupled to microtubule function through GEF-H1, thereby coordinating growth signals with changes in cell shape, migration, and/or morphogenesis. We show that the mutant GEF-H1^{A87–151}, unable to interact with the microtubule array, is largely cytoplasmic (Meiri et al., 2012) and is able to induce KSR-1 membrane translocation and ERK1/2 phosphorylation in the absence of either PDGF or oncogenic RAS. These findings suggest that the release of GEF-H1 from microtubules links HRAS^{V12} to KSR-1 function. This idea is supported by the observations that depolymerization of microtubules potently activates components of the MAPK pathway through currently unknown mechanisms (Brubkova et al., 2005; Guo et al., 2012; Hayne et al., 2000). Active HRAS contributes to microtubule instability that may promote the invasive behavior of transformed cells and reinforce the GEF-H1 positive feedback loop on the MAPK pathway (Harrison and Turley, 2001). In addition, ERK phosphorylation and activation of GEF-H1 (Fujishiro et al., 2008) might trigger its release from microtubules, where it can interact with cytosolic KSR-1. The signaling events that coordinate the spatial coupling of GEF-H1 with cytosolic KSR-1 remain to be elucidated.

The identification of GEF-H1 as a component of the RAS signaling circuitry is part of an emerging role of RhoGEFs in RAS signaling. TAM1, a Rac exchange factor, is directly activated by RAS-GTP through a RAS binding motif in its N terminus and is required for RAS-induced skin tumors (Lambert et al., 2002; Malliri et al., 2002). The RhoGEF AKAP-Lbc was shown to couple PKA to KSR-1 through its A-kinase anchoring protein scaffold function (Smith et al., 2010). GEF-H1 may also be important in other genetic contexts, because it has been reported to contribute to the growth and survival of cell lines harboring stabilizing p53 mutations and those expressing the oncogene hPPTG1 (Mizuarai et al., 2006; Liao et al., 2012).

The GEF-H1-mediated feedback loop adds to a growing number of other feedback loops that control flux through the MAPK pathway. ERK1/2-dependent phosphorylation of upstream components SOS, RAF, and EGFR (Buday et al., 1995; Porfiri and McCormick, 1996; Dougherty et al., 2005; Ritt et al., 2010; Heisermann et al., 1990; Li et al., 2008) dampens further pathway activation, and a second, kinetically slower, negative feedback loop involves the induction of DUSP phosphatases that directly...
GEF-H1 Is Required for Oncogenic RAS Signaling

A

Normal
PanIN-1B
PanIN-3
ADC

B

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Panc-1

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GEF-H1 Is Required for Oncogenic RAS Signaling

Animal Studies

All animal studies were carried out using protocols that have been approved by the University Health Network Animal Care Committee. Detailed experimental procedures are provided in Supplemental Experimental Procedures.

Cell Treatments

ER-HRAS V12 MEFs were starved in DMEM containing 0% FBS for 16 hr then treated with 100 nM 4-OHT (Sigma). For MEK and PI3K inhibition experiments, cell lines were cultured in complete medium and incubated with PD98059, U0126, or LY294002 (Sigma) diluted in DMSO (Sigma) for 48 hr. For immunofluorescence studies, MEFs were starved for 24 hr in 0% FBS and treated in DMEM containing 10 mM HEPES and 0.5 mg/ml fatty acid-free BSA (A8806, Sigma). PDGF (Sigma) was suspended in HBSS containing 0.5 mg/ml fatty acid-free BSA and 20 mM HEPES to a stock concentration of 1 mM.

Luciferase Reporter Assays

The regulatory sequence of murine Arhgef2 (nucleotides 62–1,968 upstream of the transcription start site) was PCR-amplified from mouse BAC clones and inserted into the pGL3 luciferase vector to generate pArhgef2Luc (Promega, E1910). MEFs or NIH 3T3 cells expressing empty vector, KRAS V12, or BRAG2 were plated in a 24-well plate in triplicate at 7 × 10^3 cells/well. After 16 hr, cells were cotransfected with 50 ng pArhgef2Luc, empty vector, T7-HRAS V12, or T7-KRAS V12 expression plasmids and 1 ng pHRL-SV40 (Promega) using LipoD293 (SignaGen, SL100668) or Lipofectamine 2000 (Invitrogen). Cell lines were considered statistically significant.

Immunohistochemistry

In this study, we used a human pancreatic TMA generated in a previously published study (Al-Aynati et al., 2004). The use of this TMA in this study was approved by the University Health Network Research Ethics Board (protocol 04-0018T). Immunohistochemistry was performed using the Biotin-Streptavidin-HRP detection system and a human GEF-H1 antibody (14B11 mouse monoclonal antibody) at 1:500 dilution. To evaluate the expression levels of GEF-H1, staining intensity in the ductal cells or lesions were judged by two observers. GEF-H1 protein expression was assayed by western blot using tubulin as a loading

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