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REVIEW

Dynamic Coupling of MAPK Signaling to the Guanine Nucleotide Exchange Factor GEF-H1

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Abstract: The *KRAS* gene is nearly ubiquitously subjected to activating mutation in pancreatic adenocarcinomas (PDAC), occurring at a frequency of over 90% in tumors. Mutant KRAS drives sustained signaling through the MAPK pathway to affect frequently disrupted cancer phenotypes including transcription, proliferation and cell survival. Recent research has shown that PDAC tumor growth and survival required a guanine nucleotide exchange factor for RAS homolog family member A (RhoA) called GEF-H1. The GEF-H1 protein, encoded by the *ARHGEF2* gene, is a microtubule-associated GEF for RhoA that promotes invasion-migration of PDAC cells via activation of RhoA. Unexpectedly, independent of its RhoGEF activity, GEF-H1 was found to potentiate MAPK signaling by scaffolding protein phosphatase 2A (PP2A) to the kinase suppressor of Ras 1 (KSR-1). In a feedback-dependent manner, enhanced MAPK activity drives expression of *ARHGEF2* via regulation of transcription factors ETS and SP, and the RAS responsive element-binding protein 1 (RREB1). RREB1 a negative regulator of *ARHGEF2* expression, is downregulated in PDAC cells, which permits sustained expression of GEF-H1 for PDAC tumor survival and subsequent MAPK pathway activation. Given that MAPK targeted therapies show limited clinical efficacy, highlights the need for novel targets. This review describes the unexpected complexity of GEF-H1 function leading to positive feedback that potentiates RAS-MAPK signaling and suggests inhibition of GEF-H1 as a therapeutic strategy for RAS-driven cancers.
Keywords: ARHGEF2, GEF-H1, pancreatic, RAS-MAPK, RhoA, RREB1

Introduction

The Ras family of genes is encoded by three proto-oncogenes, including *KRAS, HRAS,* and *NRAS*, which are all ubiquitously expressed and are sites of frequent mutation in human cancers.[1,2] The RAS genes encode guanosine triphosphate (GTP)-binding proteins that mediate signal transduction from receptors on the cell surface to activate signaling pathways that control the fate of transcription, cell cycle progression, cell motility, proliferation, and survival.[1–4] Gain-of-function mutation of RAS are prevalently mutated in cancer, which drives cellular transformation.[1]

Pancreatic ductal adenocarcinoma (PDAC) is a highly metastatic and deadly solid tumor with a dismal a 5-year survival rate of 5–12%, depending on the disease stage at diagnosis.[5–7] *KRAS* mutation is nearly ubiquitous in PDAC found in >90% pancreatic adenocarcinomas in all tumor grades.[1,8,9] Incidence of PDAC is increasing at a rate of ~1% per year and may become the second leading cause of cancer-related deaths by 2030.[10] PDAC patients display poor response to conventional therapy, which only prolongs survival by a few months, highlighting an unmet clinical need for novel therapeutics.[6]

Acquisition of oncogenic *KRAS* is considered an early genetic event observed in the progression model of PDAC.[7] *KRAS* mutations are demonstrable in a large percentage of early pancreatic intraepithelial neoplasia (PanIN) lesions and intraductal papillary mucinous neoplasms (IPMN), suggesting *KRAS* mutation is an initiating genetic event driving neoplasia. Loss of *CDKN2A* and the loss of *SMAD4* concomitant with mutations of *TP53* and *BRCA2* are frequently found concurrent with *KRAS* mutation, showing as intermediate and late events in the progression model, respectively.[7]

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Transformation by oncogenic RAS coincides with marked alterations to the actin cytoskeleton, including decreased stress fiber formation and focal adhesions.[11] The actin cytoskeleton is regulated by Rho small GTPases including Rho, Rac, and CDC42. The gene *RHOA* encodes the Ras Homolog Family Member A, a GTPase that modulates migration and proliferation through coordinated activity of the actin cytoskeleton and the mitogen-activated protein kinases (MAPK) to facilitate transformation by oncogenic RAS.[11–14] Recently, the MAPK-regulated phospho-proteome in PDAC revealed an assemblage of cellular processes driving tumorigenesis that integrated with Rho signaling pathways.[15] Thus, RhoA activation drives outcomes that induce stress fiber dynamics and support RAS transformation. The second outcome synergizes with elements of the MAPK pathway to stimulate cell proliferation.[11]

Despite decades of research, the mechanisms coordinating oncogenic RAS with RhoA signaling to drive tumorigenesis remain poorly understood. An important connection was established when it was discovered that a microtubule (MT)-associated guanine nucleotide exchange factor for RhoA, called GEF-H1, was found to be essential for the growth and survival of HRAS transformed cells and pancreatic tumor xenografts.[16] Independent of its RhoGEF activity, GEF-H1 was shown to have a novel scaffolding function that augmented MAPK signaling. Herein, the complex functional regulation, transcriptional control, and cancer-dependent roles of GEF-H1 are described that frame a feedback mechanism initiated by RAS, that collectively coordinates MAPK pathway activation and cell survival in PDAC. Collectively, these data support GEF-H1 as a potential novel therapeutic target that could be exploited for the treatment of PDAC.

The RAS-MAPK Pathway

The RAS-MAPK pathway is a highly conserved signal transduction cascade that regulates cell growth, survival, migration, apoptosis, and differentiation.[17–19] In normal cells, mitogenic signals activate receptor tyrosine kinases (RTKs) on the cell surface to activate RAS and the downstream activation of MAPK enzymes. Pathway activation collectively modulates diverse cellular processes triggered by growth factors, inflammatory cues, and cytokine stimulation.[19,20] Mammalian cells express four primary MAPKs: ERK1/2, c-Jun N-terminal kinase (JNK)1–3, p38, and ERK5.[20–22] Activated MAPK enzymes may regulate cytoplasmic and nuclear targets to modulate transcription factor (TF) activity influencing gene expression (Figure 1A – pathway 2).

RAS GTPases link RTK activation to the nucleus via sequential phosphorylation of Raf, MEK1/2, and ERK1/2 kinases, commonly referred to as the RAS-MAPK pathway (Figure 1A – pathway 1).[19,23–26] Through poorly understood mechanisms, RAS proteins are modified on their C-terminal CAAX motifs to enable plasma membrane localization, which is critical for function.[27] RAS activation by RTKs is coupled to additional effector pathways, including the phosphoinositide 3-kinase (PI3K)-AKT pathway, Ral guanine nucleotide dissociation stimulator (RalGDS), phospholipase Cε (PLCε)-protein kinase C (PKC), and TIAM1-Rac that relay mitogenic inputs to activation of gene expression driving pro-tumorigenic phenotypes.[28–34] Effectors downstream of RAS have overlapping targets that provide inputs for promoting crosstalk. Thus, complicating the development of therapeutic strategies, blocking one pathway can lead to the compensatory activation of others.[35]

RAS proteins are small GTPases, which function as molecular switches (Figure 1A – pathway 1), existing in active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound states.[3] Guanine nucleotide exchange factors (GEFs) promote the exchange activity of GDP for GTP leading to activation of RAS.[36] RAS proteins contain intrinsic, albeit weak GTPase activity that is augmented by RAS GTPase activating proteins (GAPs) that inactive RAS via catalytic hydrolysis of GTP.[36,37] RAS-GTP interacts directly with Raf initiating the MAPK cascade.[32] Gain-of-function mutation, as observed in cancer cells, traps RAS in a constitutively GTP bound state that culminates in sustained activation of MAPK signaling.[1,3]

Raf kinase is responsible for the activation of MEK1/2 (Figure 1A – pathway 1). The Raf family consists of three kinases (*ARAF, BRAF*, and *CRAF* or RAF1) that associate with RAS to activate downstream kinases. Raf monomers reside as autoinhibited in the cytosol.[38] Raf binds RAS directly via a RAS binding domain (RBD) in the N-terminal region.[39] Upon RAS binding, the autoinhibition of Raf is relieved, permitting the kinase domain to form homo- or heterodimers that trigger phosphorylation of the dimer components.[39,40]

MEK kinases activate ERK via phosphorylation of tyrosine and serine/threonine residues. MEK dimerization enables activation by Raf and is necessary for ERK phosphorylation.[41] Both MEK1 and MEK2 form homodimers, which aligns

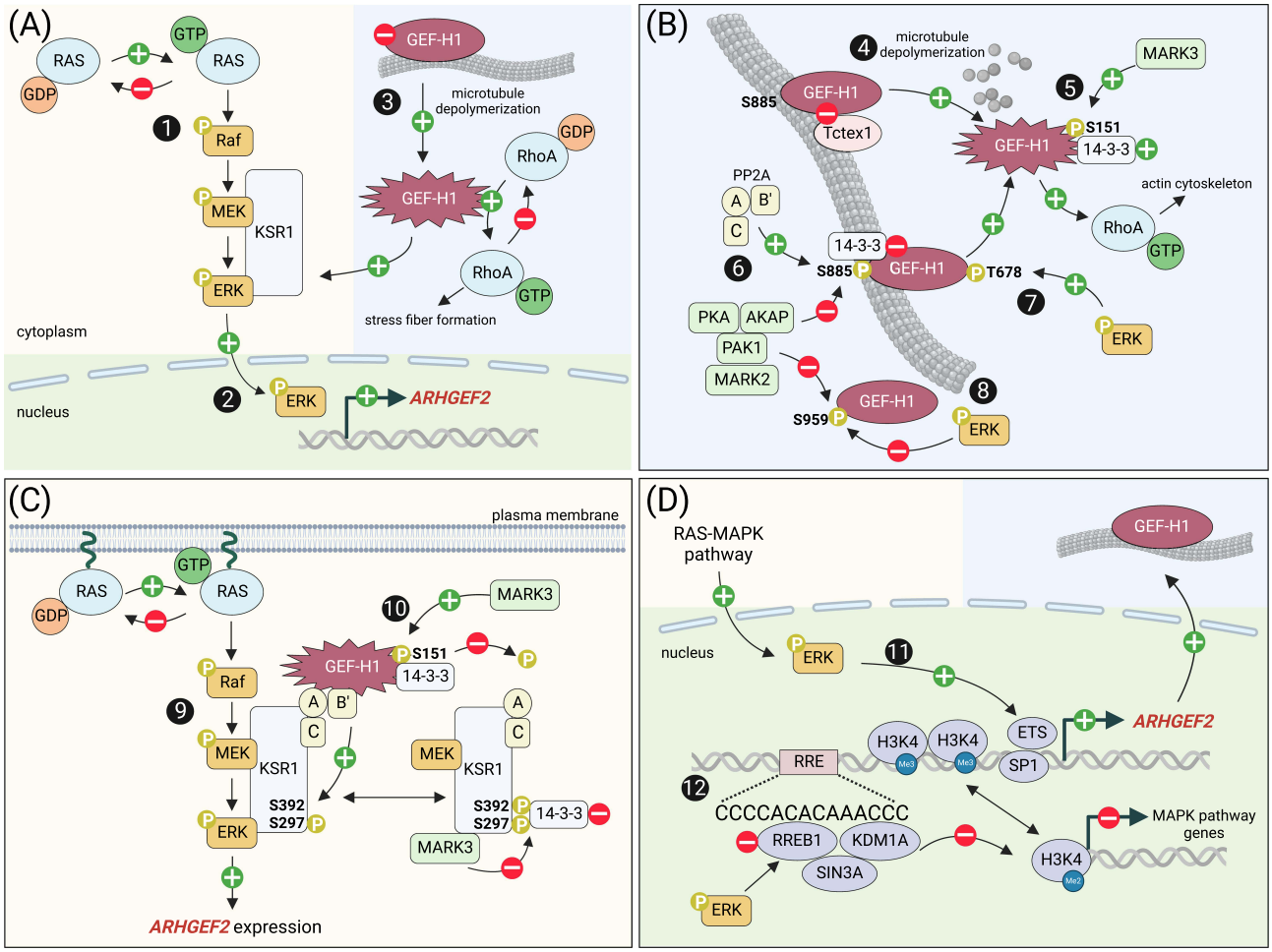

Figure 1 Signal transmission through the RAS-MAPK pathway is intricately connected to the expression of *ARHGEF2* and its gene product GEF-H1. Shown are the mechanisms demonstrated to potentiate RAS-MAPK pathway signaling by GEF-H1. The pale-orange background highlights MAPK pathway and signaling activity in cytoplasm; the blue background highlights GEF-H1/RhoA specific signaling regulation and factors; the green background highlights ERK function and gene regulation in the nucleus. The plus signs (+) indicate activation events and the minus signs (−) indicate inhibitory events. Protein phosphorylation is indicated by (P). Specific reference to pathways described in the text and figure legend are numerically delineated using square brackets in the figure legend and black circles in the figure. (**A**) Summary of RAS-MAPK pathway and GEF-H1 activation of RhoA. Canonical activation of RAS leads to sequential phosphorylation of Raf, MEK and ERK scaffolded by KSR-1 [1]. Phosphorylated ERK translocates to the nucleus and activates ARHGEF2 expression [2]. GEF-H1 is a microtubule (MT) associated GEF specific for RhoA [3]. (**B**) GEF-H1 is regulated by MT binding and phosphorylation. GEF-H1 is maintained in an inactive state by binding Tctex1 [4]. MT disassembly releases GEF-H1 to the cytosol where it may stimulate nucleotide exchange on RhoA [5]. Phosphorylation of GEF-H1 at S151 by MARK3 disrupts the Tctex1 interaction and promotes mutually exclusive 14-3-3 binding. Phosphorylation of S885 on GEF-H1 creates an inhibitory 14-3-3 binding site to maintain GEF-H1 in a catalytically inactive state. S885 is dephosphorylated by PP2A [6]. GEF-H1 is phosphorylated by ERK at S678 [7]. ERK and other kinases inhibit GEF-H1 by phosphorylation at S959 [8]. (**C**) GEF-H1 release from MTs activates the MAPK pathway. KSR-1 acts as a scaffold for MAPK pathway components to facilitate signal transmission [9]. KSR-1 is phosphorylated on S297 and S392 by MARK3 and held in an inactive state in the cytosol by 14-3-3 proteins. Following MT disassembly, GEF-H1 scaffolds the PP2A B' subunit to KSR-1 mediating dephosphorylation of S392 and activation of MAPK signaling [10]. Activation of GEF-H1 required phosphorylation of S151 by MARK3 promoting 14-3-3 binding. (**D**) *ARHGEF2* is transcriptionally regulated by the RAS-MAPK pathway. Phosphorylated ERK translocates to the nucleus to activate transcription factors ETS and SP1 on the *ARHGEF2* promoter [11]. Transcription leads to increased GEF-H1 protein. RAS inhibits *ARHGEF2* via a Ras Response Element (RRE) in the *ARHGEF2* promoter negatively regulated by the transcriptional repressor RREB1 [12]. The sequence shown is the RRE in the human *ARHGEF2* promoter. RREB1 is a phosphorylated by ERK. RREB1 recruits an epigenetic complex containing KDM1A and SIN3A to demethylate H3K4. Demethylation of H3K4 leads to transcriptional silencing of *ARHGEF2* and other MAPK genes. Methylation marks (Me2 and Me3) are shown in blue circles. Created with BioRender.com.

the activation loop of one with the catalytic center of the other.[42] In addition, MEK1 and MEK2 can heterodimerize, which has been shown to control the duration and intensity of ERK activity.[43]

The ERK kinases have a pivotal role in RAS signal transduction, exercising control over multiple features of cellular function.[21,22] The ERK1/2 kinases are serine/threonine protein kinases that occupy crucial positions in signal transduction networks relaying mitogenic signaling to the nucleus (Figure 1A – pathway 2). Aberrant ERK activation can have a significant impact on cellular function via phosphorylation of target proteins.[21,22] Like Raf and MEK, both ERK1 and ERK2 can homodimerize; however, differing from Raf and MEK, ERK heterodimers are unstable.[42]

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The RAS-MAPK pathway relies on additional proteins that facilitate signaling, including anchoring proteins, docking proteins, adapters, and scaffolds. Anchoring proteins attach to the plasma membrane and interact with effectors, such as kinases.[44,45] Docking proteins bind to RTK and GPCR receptors and are essential in cellular signaling. Adapter proteins, for example, 14-3-3 proteins, facilitate links with signaling modifiers, such as kinase suppressor of RAS 1 (KSR-1), a molecular scaffold.[44] Scaffolds coordinate kinases both spatially and temporally to augment MAPK signaling.[44,46]

GEF-H1 Is a RhoA Guanine Nucleotide Exchange Factor Associated With Microtubules

GTP-bound RAS interacts with many effectors, as mentioned above, to trigger downstream signaling pathways in response to a broad array of signals.[47] RAS directly interacts with phospholipase Cε (PLCε) to produce second messengers that activate protein kinase C (PKC) and actin regulation.[33,34] PLCε is an effector for Rho GTPase activating proteins, thus connecting RAS to the activation of Rho.[48,49]

Rho GTPase family members include *RHOA, RHOB*, and *RHOC* as well as multiple Rac and CDC42 isoforms.[50,51] Rho GTPases are activated by RhoGEFs that catalyze the intrinsically slow nucleotide exchange from GDP to GTP and are inactivated by RhoGAPs that accelerate GTP hydrolysis (Figure 1A – pathway 3). Rho GTPases regulate cellular morphology, migration, and proliferation through modulation of the actin cytoskeleton and have been shown necessary for RAS transformation.[11,13,50,51]

GEF-H1 is a large ubiquitously expressed MT-associated RhoGEF, comprising 987 amino acids. GEF-H1 is a member of approximately 70 RhoGEFs that have sequential diffuse B cell lymphoma (Dbl) homology (DH) and Pleckstrin Homology (PH) domains that catalyze nucleotide exchange.[52,53] Previously, GEF-H1 was thought to be a GEF for both Rho and Rac; however, it has been established that GEF-H1 catalyzes nucleotide exchange on RhoA, but not Rac or CDC42.[52–54] GEF-H1 has a miscellany of cellular functions that includes regulation of the actin cytoskeleton, cell morphology, cell motility, polarization, dendritic spine morphology, and cell cycle regulation.[52,54–58]

Unlike other RhoGEFs, GEF-H1 is held in an inactive state by binding to the MT array (Figure 1A – pathway 3, Figure 1B – pathway 4), via its N- and C-terminal domains.[52–54,59] Enforced expression of GEF-H1 in cultured cells localized to MTs, which suppressed activation of RhoA.[54] Both the N- and C-terminus of GEF-H1 contribute to MT binding: an N-terminal deletion of GEF-H1 (92–985) or C-terminal deletion of GEF-H1 (1–572) resulted in cytoplasmic localization.[54]

GEF-H1 binding to MTs is regulated by the dynein motor light-chain (*DYNLT1* or Tctex-1).[60] Using a minimal construct based on the murine orthologue of GEF-H1, Lfc (amino acids 87–151), it was shown that Lfc binding to MTs required Tctex-1.[60] The Tctex-1/Lfc complex required intact MTs to mediate repression, establishing that one mechanism for GEF-H1 inhibition depended on Tctex-1 anchored to MTs.[60]

MT depolymerization leads to changes in the actin cytoskeleton that affect cellular processes during development and in pathogenesis. Upon MT depolymerization, GEF-H1 is released from Tctex-1 inhibition leading to activation of RhoA (Figure 1A- pathway 3, Figure 1B – pathway 4). Not simply an on/off event, GEF-H1 release from the MT binding and activation toward exchange activity is complicated by both positive and negative phosphorylation events at serine and threonine residues on GEF-H1. Phosphorylation of GEF-H1 is mediated by several kinases (Figure 1B), creating 14-3-3 regulatory proteins binding sites, which are reversed by the PP2A phosphatase.

At least two phospho-regulatory sites on GEF-H1 are motifs for the 14-3-3 regulatory proteins. The 14-3-3 family are phospho-binding proteins controlling signaling pathways critical to cellular activity including apoptosis, cell cycle progression, autophagy, glucose metabolism, and cell motility.[61] The 14-3-3 proteins recognize phosphorylated serine or threonine within a consensus motif on the target protein. Functionally, 14-3-3 binding can conceal localization signals, block phosphatase action, or modulate protein conformation into active or inhibited states.[61]

The amino acid sequence of GEF-H1 encompassing S151 is a conserved 14-3-3 binding site. GEF-H1 S151 is phosphorylated by the microtubule affinity regulating kinase 3 (MARK3).[62] MARK kinases include four family members, MARK1 (PAR-1c), MARK2 (PAR-1b or EMK), MARK3 (PAR-1a or C-TAK1), and MARK4 (PAR-1d or

MARKL1). The MARK kinase family triggers MT instability by phosphorylating microtubule-associated proteins, leading to rapid detachment that regulates the cell cycle and cell polarity.[63]

GEF-H1 phosphorylation by MARK3 was shown to antagonise binding to Tctex-1 via direct competition with 14-3-3 binding (Figure 1B – pathway 5). Upon 14-3-3 binding to phospho-S151, GEF-H1 is displaced from Tctex-1 inhibitory binding enabling RhoA activation of stress fiber and focal adhesion formation.[62] It has been shown that 14-3-3 and Tctex-1 bind GEF-H1 in a mutually exclusive manner; specifically, the interaction between GEF-H1 and 14-3-3 inhibits binding with Tctex-1.[64]

GEF-H1 is inhibited by phosphorylation of S885, which is a dominant 14-3-3 binding site.[64] 14-3-3 recognizes GEF-H1 phospho-S885, which relocates the exchange factor to the MTs independent of Tctex-1.[64,65] GEF-H1 is phosphorylated on S885 by diverse kinases, which include protein kinase A (PKA), p21-activated-kinase 1 and 4 (PAK1, PAK4), Aurora kinase A, and MARK2, to activate 14-3-3 mediated sequestration of GEF-H1 to MTs with concomitant loss of GEF activity.[64–68] GEF-H1 phosphorylation at S885 also antagonizes the interaction with Tctex-1.[64] Thus, GEF-H1 is inhibited by MT binding in a Tctex-1 dependent manner and by a mutually exclusive mechanism of MT sequestration via 14-3-3 binding phospho-S885.

GEF-H1 S885 is dephosphorylated by PP2A to release GEF-H1 from the MT array thus promoting exchange activity (Figure 1B – pathway 6). Interestingly, GEF-H1 dephosphorylation at S885 augments Tctex-1 binding, indicating different competing mechanisms exist for activation and inhibition of GEF-H1 activity.[64] Further, dissociation from MTs alone was not enough to activate GEF-H1, but also required the dephosphorylation of S885 by PP2A.[69] Collectively, GEF-H1 phosphorylation on S885 regulates RhoA-dependent actin cytoskeletal reorganization and stress fiber formation.[65]

GEF-H1 Regulation and Function Is Coupled to MAPK Activation

ERK activation has been shown to coincide with decreased MT stability, leading to enhanced cellular proliferation and invasion by activating Rho.[70] ERK directly phosphorylates GEF-H1 on threonine 678 (T678) and serine 959 (S959) to both enhance or inhibit GEF activity depending on the context, leading to activation or suppression of RhoA and regulation of proliferation and motility.[52,71,72]

ERK phosphorylates GEF-H1 at T678 (Figure 1B – pathway 7), enhancing exchange activity.[71] ERK mediated T678 phosphorylation of GEF-H1 in response to TNF-α stimulation in tubular epithelial cells triggers elevated phosphorylated MLC, a downstream RhoA effector, leading to stress fiber formation.[71] The GEF-H1 phosphorylation on T678 by ERK was shown to be complement regulated and affected subcellular localization of GEF-H1, changing a cytosolic distribution with colocalization on the MTs to a perinuclear localization.[73] The change in subcellular localization was not affected by mechanical stimuli or mediated by the MT array suggesting additional mechanisms of GEF-H1 regulation that are not completely understood. Future research may help establish how ERK phosphorylation of GEF-H1 affects MT binding in a context-specific manner.

ERK inhibits GEF-H1 by phosphorylating S959 (Figure 1B – pathway 8), a site also targeted by PAK, Aurora A, Cdk1, and MARK2 kinases, highlighting S959 phosphorylation is critical in regulating GEF-H1 activity.[52,65,66,74] The phosphorylation of GEF-H1 on S959 by ERK suppressed GEF activity impacting invasion and migration of cancer cells.[74] HCT116 cells treated with MEK inhibitors displayed decreased invasiveness accompanied by a change in mesenchymal morphology indicative of high RhoA activity and suggestive of EMT-related mechanisms.[74] The effect was attributed to GEF-H1 dephosphorylation on S959, which led to increased GEF-H1 activity. Research suggests the inhibition of GEF activity resulting from S959 phosphorylation may be mechanistically distinct from inhibition of GEF-H1 by MT binding.[74] These results further support context-specific phosphorylation of GEF-H1 and that ERK deactivation of GEF-H1 may be distinct from inactivation by MT sequestration. Indeed, both MAPK activity and MT reorganization are important for mitotic entry.[58,75,76]

Activation of ERK is connected to disruption of the MT array, which at one point was hypothesized to involve GEF-H1.[70,77] The connection was made conclusive when it was demonstrated that mouse embryonic fibroblasts (MEFs) expressing HRAS-V12 with genetic depletion of *Arhgef2*, the murine gene encoding the murine orthologue of GEF-H1, had significantly impaired MEK and ERK phosphorylation compared to controls. The defect in signaling was restored by

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enforced expression of human GEF-H1.[16] The decreased MEK/ERK activation observed in HRAS-V12 *Arhgef2-/-* MEFs could not be restored by expressing either AKAP-Lbc, the GEF family member most closely related to GEF-H1, or p115 RhoGEF, another member of the RhoGEF family. Further, MAPK activation was enhanced by a constitutively active GEF-H1 lacking the MT binding domain (Δ87-151) and by a catalytically inactive mutant of GEF-H1 (E243K).[16] These results confirmed a GEF-H1 dependent mechanism of MAPK activation in *HRAS-V12* cells independent of GEF catalytic activity. The data suggested that GEF-H1 may scaffold components of the MAPK pathway.

As mentioned earlier, scaffolds for the MAPK pathway coordinate kinases both spatially and temporally to augment signaling.[44,46] In mammalian cells, KSR-1 is a scaffold that functions to control flux through the MAPK pathway.[78] KSR-1 assembles B/CRAF, MEK1/2, and ERK1/2 (Figure 1C – pathway 9), facilitating efficient signal transmission necessary for RAS-mediated signaling.[78–84] Both MEK and ERK interact directly with KSR-1, in contrast, Raf may have an indirect interaction.[85] In addition, KSR-1 retains ERK in proximity to cellular effectors.[79,86] Importantly, data has demonstrated that KSR-1 activation of ERK required GEF-H1. First, a physical interaction between GEF-H1 and KSR-1 using immunoprecipitation with recombinant protein fragments of KSR-1 confirmed a direct interaction with the DH domain of GEF-H1.[16] Second, in *Ksr1-/-* MEFs, constitutively active GEF-H1 (Δ87-151) was unable to rescue ERK phosphorylation without exogenously expressed *KSR-1*.

When quiescent, KSR-1 is phosphorylated on S297 and S392 by MARK3 and maintained in an inactive state in the cytosol by 14-3-3 binding (Figure 1C - pathway 9).[85,87] MARK3 constitutively associates with KSR-1 to confer 14-3-3 binding to phospho-S392 sequestrating KSR-1 in the cytoplasm in unstimulated cells.[85,88] RAS activation prompts dephosphorylation of S392, which is the signal required to translocate KSR-1 to the plasma membrane. Following dephosphorylation, KSR-1 translocates to the plasma membrane where it engages RAS and Raf to stimulate MAPK pathway activity.[78] Unexpectedly, translocation of KSR-1 to the plasma membrane depended on GEF-H1 release from the MTs, indicating that GEF-H1 in addition to binding KSR-1, was functionally required for activation of signaling via KSR-1.[16]

In HRAS-V12 *Arhgef2-/-* MEFs, the constitutively active S392A mutant of KSR-1 was sufficient to restore ERK phosphorylation in the absence of GEF-H1.[16] This result suggested GEF-H1 binding KSR-1 facilitated dephosphorylation of S392. Genetic studies have shown that KSR-1 is positively regulated by the PP2A phosphatase to mediate RAS signaling.[89,90] PP2A is an essential phosphatase needed to dephosphorylate KSR-1 at S392 in response to RAS signaling.[87] PP2A is a heterotrimeric serine/threonine protein phosphatase composed of structural (A), regulatory (B), and catalytic (C) subunits. The A and C subunits are constitutively associated with each other and with KSR-1 in a core complex that enables binding of the B subunit.[91] In mammals, there are four B subunits (B, B′, B″, and B‴) that establish substrate specificity of the holoenzyme.[91] It is the recruitment of the B′ subunit to KSR-1 that is coupled to RAS activation via a previously unknown mechanism.[87]

Using immunoprecipitation, GEF-H1 was shown to be the missing piece, interacting with PP2A subunits A and C, and co-purifying with the B′ regulatory subunits.[16] Using truncating constructs of GEF-H1 for immunoprecipitation experiments, it was found that the B′ subunit interacts with GEF-H1 via the PH domain.[16] The PP2A holoenzyme also dephosphorylates GEF-H1 at phospho-S151 to restore MT binding, ostensibly mediated in part by direct proximity of the B′ subunit with GEF-H1.[62] Therefore, although yet to be experimentally validated, the regulation of S151 on GEF-H1 may provide an intrinsic signal critical to its ability to activate KSR-1 (Figure 1C – pathway 10). An unanswered question remains, whether GEF-H1 scaffolding the B′ subunit to KSR-1 and MAPK activation is permissive with or mutually exclusive with MARK3-GEF-H1 binding and cell polarization.[62] In summary, GEF-H1 scaffolds the B′ subunit of PP2A to KSR-1, facilitating the dephosphorylation of KSR-1 on S392 and enhancing MAPK signaling.

GEF-H1 Is Encoded by *ARHGEF2*, A Transcriptional Target of RAS

As described above, GEF-H1 acts as a scaffold to potentiate MAPK signaling via KSR-1 and thus coordinate the transcription of MAPK regulated genes. In *HRAS-V12* transformed cells, increased GEF-H1 mRNA and protein was observed relative to control cells, suggesting a feedback loop where MAPK activation by GEF-H1 triggers transcriptional activation of *ARHGEF2*, the gene that encodes for GEF-H1.[16] Indeed, previous research has corroborated *ARHGEF2* as a transcriptional target of oncogenic RAS signaling (Figure 1A – pathway 2).[16,92]

The human *ARHGEF2* gene spans over 30 kb on chromosome 1. Based on phylogenetic conservation and CpG island enrichment, a 1.9 kb region proximal to the first exon of murine *Arhgef2* was shown to contain promoter activity.[16] Mapping this sequence onto the human genome aligned with the transcription start site (TSS) of the human *ARHGEF2* near a histone 3-lysine 27 acetylation (H3K27Ac) element, indicative of active chromatin, thus implicating this location as the human *ARHGEF2* proximal promoter.

Experimentally, this genomic region was validated for promoter activity using a series of genomic fragments cloned into a luciferase reporter plasmid. Several fragments demonstrated robust luciferase activity in PDAC cell lines indicative of functional promoter elements.[92] A minimal human promoter sequence was identified containing a conserved region between −264 and 23 relative to the TSS, which was demonstrated to be RAS responsive.[92] A panel of small molecules inhibiting RAS effector pathways confirmed that transactivation of the *ARHGEF2* minimal promoter was suppressed by inhibiting MEK1/2, thus connecting GEF-H1 expression to the RAS-MAPK pathway. The minimal promoter was also found to be responsive to multiple inputs, including activation by JNK, Rac, and TGF-β and inhibition through p38-MAPK and Rho.[92] The contribution of these various signalling pathways to the sustained expression of GEF-H1 in PDAC is yet to be determined.

An in silico analysis of TF binding sites was performed on the *ARHGEF2* minimal promoter sequence to discover potential MAPK regulated TFs driving promoter regulation. The *ARHGEF2* promoter was experimentally determined to be regulated by several TFs known to be targets of ERK, including ETS family members ETS1 and ELK1, SP family members SP1 and SP3, and the RAS responsive element-binding protein 1 (RREB1).[92]

The ETS family, which includes ELK1 and ETS1, is a family of 28 genes in humans that are crucial for development, proliferation, and transformation.[93] Gene regulation by the ETS proteins converge on diverse signaling networks, including MAPK, p38, JNK, and PI3K consistent with regulatory inputs described to activate the *ARHGEF2* promoter. Experimental data confirmed that *ARHGEF2* is transactivated by both ELK1 and ETS1 (Figure 1D – pathway 11).[92,93]

The *ARHGEF2* promoter contained four GC boxes, which are binding sites for the specificity proteins (SP).[94] The SP are zinc finger TFs with over 16 members known to regulate GC-rich elements found in promoters, enhancers, and locus control regions.[94] Specifically, two SP factors, SP1 and SP3, are both phosphorylated by ERK,[95] and were validated to regulate *ARHGEF2* expression.[92,96] Interestingly, SP1 has been found upregulated in PDAC cell lines and surgically resected PDAC tumors and regulates proliferation of pancreatic cancer cells.[97–99]

The *ARHGEF2* promoter contained a highly conserved RAS response element (RRE), a binding site for RREB1 (Figure 1D – pathway 12). RREB1 is a large zinc finger TF that has been demonstrated to activate and inhibit transcription in response to RAS signaling.[92,100–104] The regulation of RREB1 by ERK to mediate its transcriptional activity has been previously described.[104,105] In *RREB1* knockdown cells, the *ARHGEF2* minimal promoter drove robust expression of luciferase demonstrating *ARHGEF2* is negatively regulated by RREB1.[92] Further, promoter activation was recapitulated using a minimal promoter variant in which the RRE was disrupted by mutation, thus validating direct regulation by RREB1.[92]

In PDAC, *RREB1* expression decreases during PanIN development and has low expression in PDAC patient derived xenografts.[92,106] The down regulation of *RREB1* in PDAC correlates with *ARHGEF2* promoter activation observed in cell lines concomitant with activation of RhoA and MAPK signaling.[92,107] Transient *RREB1* knockdown in PDAC cell lines significantly increased *ARHGEF2* mRNA and protein expression, which affected the amplitude and duration of RhoA GTP activation.[92] *RREB1* knockdown caused increased activation of ERK dependent on the sustained expression of GEF-H1.[92] A combination of chromatin immunoprecipitation and RNA sequencing revealed the RREB1 transcriptome, in addition to *ARHGEF2*, included MAPK pathway components *HRAS, MAP2K2* which encodes for MEK2, and *FGFR4*.[100]

Recent research has established that RREB1 exerts epigenetic control over RRE containing promoters.[100] RREB1 BioID enabled the discovery of an epigenetic complex containing the histone demethylase KDM1A, HDAC, and other transcriptional regulators including switch-insensitive 3 family member A (SIN3A) regulated by RREB1 to suppress transcription.[100] *RREB1* downregulation, as occurs in PDAC, caused RRE promoters to be marked more active via increased histone 3-lysine 4 (H3K4) trimethylation, which leads to increased transcription (Figure 1D – pathway 12). The *ARHGEF2* promoter was negatively regulated by RREB1 in an H3K4 methylation-dependent manner. Genetic disruption

of *RREB1* expression led to increased MAPK pathway activity via transcriptional dysregulation of multiple genes including *ARHGEF2*.[100] Collectively, these disparate results demonstrate that oncogenic RAS can promote increased cellular viability in PDAC by upregulating the expression of GEF-H1 though a transcriptional program activated by the MAPK pathway.

The Role of GEF-H1 in Cancer Has Potential for Targeted Therapy

Until recently, activating mutations for RhoA family proteins in cancer have not been identified,[108] suggesting that activation of RhoGEFs contribute to cancer initiation and progression.[109] Multiple studies have shown that RhoGEFs are oncogenic, and their expression levels increase with malignancy.[109–111] Previously, the oncogenic potential of GEF-H1 was characterized using Lfc in a screen for novel proteins capable of inducing oncogenic transformation in murine fibroblasts.[112] A truncated version of GEF-H1 lacking the microtubule-binding domain was shown to have oncogenic potential in fibroblast transformation assays and to induce tumor formation in mice.[113]

In 10 of 25 mutant *KRAS* PDAC cell lines (40%), GEF-H1 contributed to both cell growth and survival with little effect on cell lines harboring wildtype *KRAS*.[16] Genetic depletion of *ARHGEF2* negatively affected PDAC cell survival in vitro and hindered the growth of pancreatic xenografts in vivo, supporting a critical functional role of GEF-H1.[16,114] In HRAS-V12 transformed NIH 3T3 cells, *ARHGEF2* supported cell growth and survival; specifically, loss of *ARHGEF2* inhibited HRAS-V12-induced colony formation in agar and xenograft tumor formation.[16] Several PDAC cell lines demonstrated a range of *KRAS* oncogene addiction which correlated to an *ARHGEF2* dependency index.[107] For example, MiaPaCa-2, a PDAC cell line with an activating *KRAS* G12C mutation, is exquisitely sensitive to the sustained expression of *ARHGEF2*. Knock down of either *KRAS* or *ARHGEF2* set off an apoptotic response in these cells.[107]

GEF-H1 has a demonstrated role in many cancer types driving activation of RhoA signaling to induce pro-proliferative and metastatic potential.[115–117] Activating mutations of GEF-H1 are not commonly found drivers of cancer, suggesting upregulation of *ARHGEF2* may contribute to cancer initiation and progression. Transactivation of *ARHGEF2*, leading to increased GEF-H1 protein levels, correlates with tumor progression in PDAC.[16] Indeed, mutant *KRAS* colon cancer cell lines transfected with GEF-H1 showed increased viability, reduced apoptosis, and accelerated cell cycle progression.[118] Establishing a distinct role for GEF-H1 in RAS transformation and tumor initiation is an exciting area of continued research.

Clinical data support that *ARHGEF2* overexpression is associated with more aggressive tumor phenotypes and shorter recurrence-free survival.[116] Increased expression of *ARHGEF2* correlates with poor prognosis in patients with PDAC.[116] Following surgery, circulating pancreatic tumor cells display a cell motility gene signature with increased levels of *ARHGEF2* shown to negatively predict overall survival.[119] *ARHGEF2* was found upregulated in patient colon cancers where, it may promote metastasis via the activation of RhoA.[118]

In gastrointestinal stromal tumors treated with Imatinib, an RTK inhibitor, *ARHGEF2* was among several genes showing altered response after treatment suggesting that *ARHGEF2* expression might be a promising therapeutic target for susceptible cancers.[120] Direct pharmacological targeting of GEF-H1 is yet to be described, however, several lines of evidence implicate small molecule inhibition of MT dynamics as promising cancer therapeutics, indirectly affecting GEF-H1 activity and cellular response. For example, paclitaxel caused polymerization and stabilization of MTs, showing efficacy in ovarian and breast cancer, malignant melanoma and other solid tumors.[121] Adjuvant chemotherapy after surgical resection with a combination of gemcitabine and paclitaxel can significantly enhance the prognosis for patients with advanced pancreatic cancer.[122] Targeting both MT stability and the MAPK pathway is another potential approach. For example, a phase-II clinical trial found that combining docetaxel with the MEK inhibitor selumetinib enhanced survival in patients with KRAS-driven non-small cell lung cancer.[123] Unfortunately, the side effects of paclitaxel chemotherapy are severe, arguing for the need of a specific inhibitor of GEF-H1 function.

Plinabulin is a class of small molecule that inhibits tubulin polymerization. Derived from a natural product to disrupt tubulin depolymerization activity, the anticancer activity of plinabulin inhibits PDAC cell growth and is undergoing clinical trials.[124,125] Although plinabulin inhibition of MT polymerization may activate GEF-H1 based on the mechanisms described herein, the benefit was mediated by blocking endosomal recycling. Following plinabulin treatment, KRAS accumulated in endosomal vesicles causing a potent inhibition of PI3K/AKT signaling. Interestingly, AKT inhibition was

accompanied by an increase in MAPK activity, which potentially could be GEF-H1 mediated.[125] Gemcitabine in combination with MBRI-001, a synthetic derivative of plinabulin, induced apoptosis by increasing DNA damage response with significantly superior tumor suppressing activity in two PDAC cell lines compared to each agent alone.[126] Although these results are promising, the complex interplay between GEF-H1/RhoA and RAS-MAPK demands future research to establish if combination therapy would benefit more from inhibition of MAPK and MT.

Conclusions and Future Perspectives

Aberrant MAPK signaling plays a central role in tumorigenesis, leading to initiation and progression of many human cancers.[3] Despite significant efforts to decode molecular and cellular mechanisms of RAS transformation, the complexity of downstream effectors and signalling cascades regulated by RAS remain to be completely understood. Undoubtably, future research into how KRAS drives sustained cell growth and cellular transformation will facilitate new therapeutic strategies. Significant new effort is being applied to elucidate the MAPK pathway resistance mechanisms in PDAC.[127–130] Targeting GEF-H1 directly or blocking activation of *ARHGEF2* expression may lead to novel approaches for RAS-dependent cancer treatment.

For decades, the MAPK pathway has been the focus of targeted therapeutics to inhibit RAS-mutant tumors with limited success in the clinic. Based on the work presented in this review, if a specific inhibitor of GEF-H1 could be engineered, the potential for combination therapy with RAS inhibitors and/or MAPK inhibition would be a compelling strategy. For example, AZD6244 is a highly selective allosteric MEK1/2 inhibitor but exhibits modest clinical activity alone showing better efficacy in combination therapies.[131] AZD6244 in combination with the MT stabilizing agent paclitaxel led to tumor regression via deactivation of GEF-H1 in human tumor xenograft models, prompting investigation in clinical trials.[132]

In conclusion, GEF-H1 plays an essential role in RAS activation of the MAPK pathway that coordinates mitogenic signals and MT sensing with cellular phenotypes. Currently, there is a paucity of personalized therapies to achieve better treatment outcome for PDAC. Delineating the molecular mechanisms of tumor progression driven by GEF-H1 may provide a starting point for novel precision medicine.

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Disclosure

The authors report no conflict of interest in this work.

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