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RhoGEFs in Cell Motility: Novel Links Between Rgnef and Focal Adhesion Kinase

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Abstract: Rho guanine exchange factors (GEFs) are a large, diverse family of proteins defined by their ability to catalyze the exchange of GDP for GTP on small GTPase proteins such as Rho family members. GEFs act as integrators from varied intra- and extracellular sources to promote spatiotemporal activity of Rho GTPases that control signaling pathways regulating cell proliferation and movement. Here we review recent studies elucidating roles of RhoGEF proteins in cell motility. Emphasis is placed on Dbl-family GEFs and connections to development, integrin signaling to Rho GTPases regulating cell adhesion and movement, and how these signals may enhance tumor progression. Moreover, RhoGEFs have additional domains that confer distinctive functions or specificity. We will focus on a unique interaction between Rgnef (also termed Arhgef28 or p190RhoGEF) and focal adhesion kinase (FAK), a non-receptor tyrosine kinase that controls migration properties of normal and tumor cells. This Rgnef-FAK interaction activates canonical GEF-dependent RhoA GTPase activity to govern contractility and also functions as a scaffold in a GEF-independent manner to enhance FAK activation. Recent studies have also brought to light the importance of specific regions within the Rgnef pleckstrin homology (PH) domain for targeting the membrane. As revealed by ongoing Rgnef-FAK investigations, exploring GEF roles in cancer will yield fundamental new information on the molecular mechanisms promoting tumor spread and metastasis.

Keywords: Cell motility, Dbl-related GEF, FAK, integrin signaling, Rgnef/ARHGEF28, RhoGTPase.

INTRODUCTION

Cell motility is a complex process that involves cellular interactions with the environment leading to intracellular changes that modulate protein function and gene expression [1, 2]. Communication between the outside and inside of cells is relayed from the extracellular matrix (ECM) *via* integrins to the actin cytoskeleton [3, 4]. Signals initiated from inside cells can also alter integrin activation states to modulate cell adhesion to the ECM [5]. All of these changes must be coordinated in time and space within cells in order to initiate and maintain directional movement [6].

The Rho family of GTPases are small ubiquitous (~21 kDa) signaling G proteins (guanine nucleotide-binding proteins) that bind to and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). Canonical members include RhoA, Rac1, and Cdc42 [7]. Rho-family GTPases act as switches; when they bind GTP, they are active, and, when they bind GDP, they are inactive. When bound to GTP, Rho-family GTPases associate with a variety of target proteins that regulate many aspects of intracellular actin dynamics needed for cell movement [8]. Since basal nucleotide exchange and intrinsic hydrolysis are slow, the Rho-family GTPase activation cycle is controlled in part by GTPase activating proteins (GAPs) that stimulate GTP hydrolysis and guanine-nucleotide exchange factor (GEFs) that promote the exchange of GDP for GTP [9]. The large number of GEFs and GAPs (>70 members each) far outnumber Rho GTPase targets and this likely reflects signaling diversity in Rho GTPase regulation [10]. The molecular regulation of various GEFs or GAPs contains both conserved and unique protein-specific elements. There have been recent reviews on GAPs in signal termination [11] and in the regulation of membrane traffic [12]. Herein, we will focus on GEFs.

There are two distinct GEF families for Rho proteins: those of the diffuse B-cell lymphoma (Dbl) and dedicator of cytokinesis (Dock) families [10, 13, 14]. In the interest of space and to provide a focused review, emphasis will be on the Dbl GEFs. The Dbl-homology (DH) domain (~200 amino acids) comprises a region with GEF activity and there are more than 70 human DH-containing proteins (Table **1**) [15]. The DH domain may have considerable amino acid divergence between GEFs, but it comprises a related three-dimensional structure [16]. The majority of Dbl family proteins have a DH domain followed by a pleckstrin homology (PH) domain (~100 amino acids) that binds phospholipids and other proteins [17, 18]. The conservation of the tandem DH-PH organization implies a conserved function within GEFs, but the PH domain is also found in many other human proteins [19]. In a small subset of Dbl members, the DH domain

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is followed by a BAR (Bin–Amphiphysin–Rvs) domain that can promote either protein dimerization or membrane binding [15]. Outside of the DH-PH region, GEFs encompass a diverse range of sequence motifs and domains that can connect GEFs to various subcellular sites or signaling pathways. The fact that there are greater numbers of GEFs than RhoGTPases suggests that signal integration and specificity for Rho activation may be regulated by GEF activity. Many GEFs have distinct domains that may allow for additional functional specificity. In the following discussion, we will emphasize those GEFs that contribute to the complex process of cell migration. In particular, we highlight Rgnef, a Dbl family RhoGEF that uniquely binds FAK, a well-known mediator of cell motility.

RGNEF (p190RHOGEF/ARHGEF28) AND FAK

Rgnef (previously named p190RhoGEF for its 190 kDa molecular weight, gene name Rgnef recently changed to Arhgef28) is a ubiquitously-expressed DH-PH-containing GEF [20] that can activate RhoA and RhoC in cells [21, 22]. Rgnef is most highly related to p114 (ARHGEF18), Lbc (ARHGEF13), and GEFH1 (ARHGEF2). Rgnef contains several potential regulatory motifs (Fig. **1A**), including an N-terminal leucine-rich region and a cysteine-rich zinc finger domain. The large C-terminal region of Rgnef contains a potential coiled-coil domain that can bind microtubules [21], the 3'-untranslated region of neurofilament mRNA [23], and phosphorylation independent associations with 14-3-3 [24] or c-Jun amino-terminal kinase interacting protein-1 [25]. The original sequencing of murine Rgnef contained a frame shift error that altered the coding sequence for the last 36 amino acids [20] (Protein: NP_036156, Nucleotide: NM_012026). This region is homologous to human Rgnef (GeneID 64283, NM_001080479) and as noted in a prior review [26], Rgnef contains a consensus PDZ-binding motif (IVYL) at the C-terminus, a feature shared by a subset of other GEFs [27]. One unique feature of Rgnef is that it can bind directly to focal adhesion kinase (FAK) and this interaction is dependent upon a short Rgnef peptide region (1292-1301) near the coiled-coil domain [28, 29].

FAK is a cytoplasmic protein-tyrosine kinase that is recruited to and activated at cell adhesion sites termed focal adhesions [30]. FAK acts downstream of various growth factor and integrin receptors in the control of cell shape and cell-cell adhesion changes needed for efficient cell movement [31]. Although a variety of FAK-associated signaling pathways have been characterized through analyses of FAK knockout mice/cells [32], FAK kinase-dead knockin mice/cells [33-35], and pharmacological FAK inhibition [36], the mechanisms associated with FAK recruitment and activation at receptor sites remains unclear. The tightly controlled process of cell migration involves many precise spatiotemporally regulated molecules. Since both FAK and the Rgnef effector RhoA have been shown to play significant roles in migration, the direct interaction of these two proteins likely confers an additional layer of regulation. Thus, the interaction between Rgnef and FAK is important as this provides a point of integration for the generation of contractile forces and activation of signaling cascades regulating cell movement [29]. Moreover, emerging evidence supports the importance of Rgnef-FAK interactions in promoting tumor progression [37]. In this review, we will expand upon a novel concept that Rgnef also functions as a scaffold in a GEF-independent manner to enhance FAK activation downstream of integrins [38] and how this may impact tumor biology.

DEVELOPMENT: POTENTIAL COMPENSATION BETWEEN GEFS FROM KNOCKOUT STUDIES

Regulated cell movement is a fundamental process during multicellular animal development. From C. *elegans* to primates, tissue formation results from the orchestrated migration of various cells during gastrulation, organogenesis, vasculogenesis, and neuronal pathfinding [39, 40]. Rho GTPases are key regulators of cell motility and therefore, it is not surprising that inactivation results in developmental abnormalities. RhoA, RhoB, and RhoC are related and RhoA knockout in mice leads to embryonic lethality whereas loss of RhoB or RhoC result in milder phenotypes [41-43]. These results suggest a fundamental role for RhoA whereas RhoB and RhoC may have overlapping and tissue- or disease-specific roles apart from activating common RhoA targets. Since there are ~3 times as many GEFs that activate Rho-family GTPases [10], a major challenge in the field is to understand how temporal and spatial activation of GEFs relates to RhoA activation and cell function. A standard approach is to analyze the effect of loss of expression in a transgenic mouse model. However, few developmental defects have been observed in mice lacking RhoGEFs [44, 45]. This may be attributable to either redundancy during development or tissue-specific RhoGEF expression.

Analyses of heterozygous crosses of transgenic Rgnef knockout mice showed that Rgnef-/- mice were present at normal Mendelian ratios on embryonic day 13.5 [44]. However, Rgnef-/- mice were born at a significantly lower Mendelian frequency. At birth, Rgnef-/- mice exhibit an overall smaller size than Rgnef+/- or Rgnef+/+ littermates. Analyses of Rgnef-/- offspring did not reveal apparent tissue abnormalities and this size difference was negligible by 6 to 8 weeks of age. It is likely that there is an important role for Rgnef in mouse growth or development, but that some type of partial redundancy or compensation may be occurring to lessen or bypass the potential restriction point between embryonic day 13.5 and birth. Highest Rgnef expression was found in the brain, ovary, and spleen of 10 week old mice [44]. Although roles for Rgnef have been proposed in neuronal [23, 46, 47] and immune cell [48, 49] function, Rgnef-/- mice are fertile and do not exhibit obvious defects. Moreover, partial

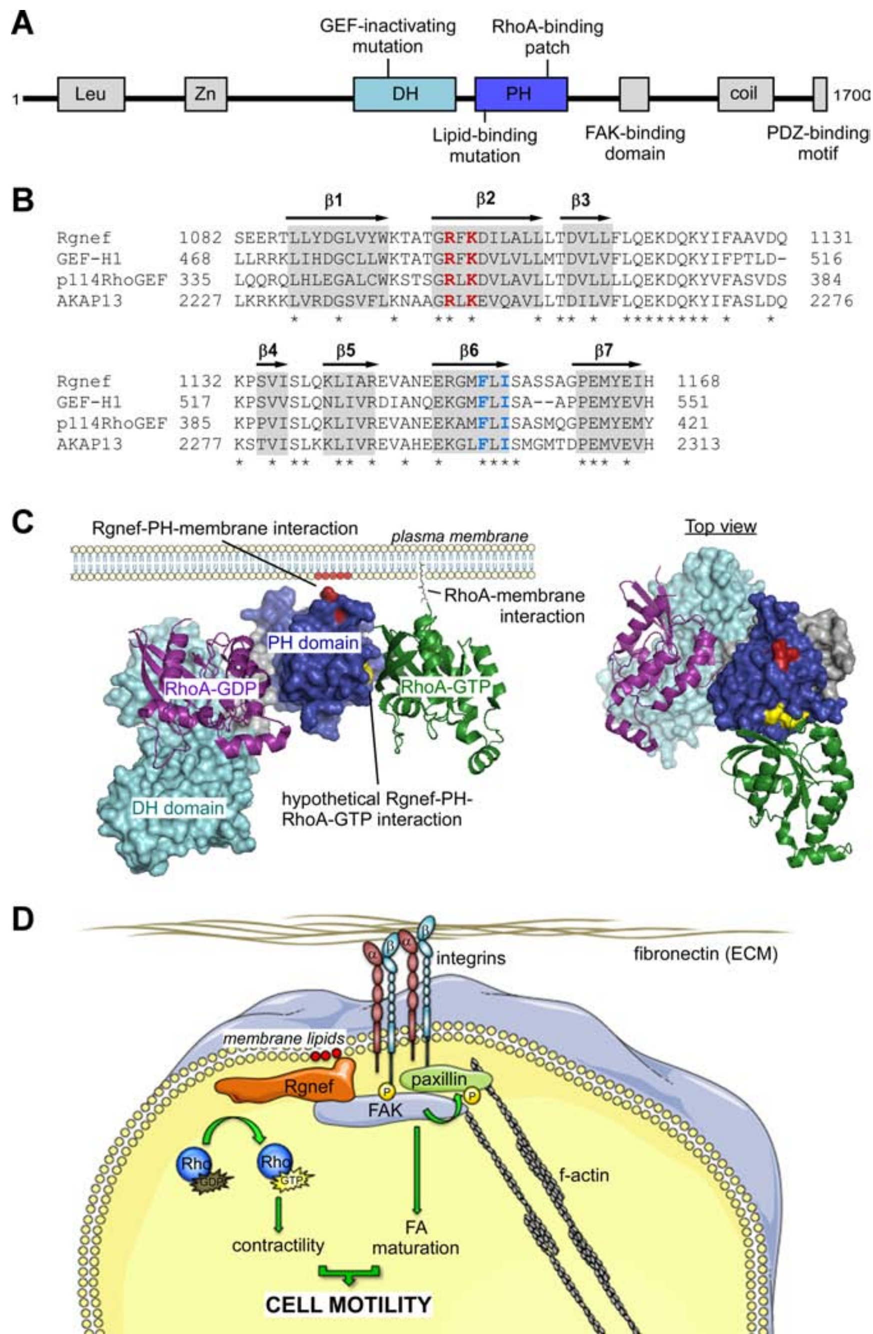

Fig. (1). Rgnef protein domains and structure. (**A**) Mouse Rgnef protein schematic. Shown are the leucine-rich domain (Leu), zinc-finger motif (Zn), tandem Dbl-homology (DH) pleckstrin-homology (PH) domain, FAK-binding domain (1292-1301), coiled-coil domain (coil), and PDZ-binding motif. Also shown are the locations of the GEF-inactivating mutation (Y1003A), lipid-binding mutation (R1098A/K1100A), and RhoA-GTP binding residues (A1151/A1153). (**B**) PH domain alignment of Lbc RhoGEF subfamily members. Highlighted in gray are putative locations of beta-strands (b1-b7), asterisks indicate identical residues. In red is the location of residues necessary for efficient PI lipid binding in Rgnef. In blue are residues necessary for binding to activated RhoA across all Lbc subfamily GEFs. (**C**) Left, theoretical structure of the Rgnef DH-PH domain at the plasma membrane. Rgnef binds to PI lipids (red) at the plasma membrane through conserved residues in the PH domain (residues in red, PH domain in blue). Rgnef also potentially binds to RhoA-GTP (green) at the plasma membrane through conserved hydrophobic residues (yellow) in the PH domain. These factors potentially localize and orient Rgnef for its GEF activity towards RhoA-GDP (purple) through the DH domain (cyan). Right, top down view of Rgnef in complex with RhoA-GTP and RhoA-GDP. Theoretical Rgnef DH-PH model created in Swiss-Model. RhoA-GDP crystal structure from PDB 1X86. RhoA-GTP crystal structure from PDB 3KZ1. Theoretical Rgnef DH-PH model created in Swiss-Model based on PDB 3KZ1 [97] (**D**) Simplified model of Rgnef function downsteam of integrin signaling. Cell binding ECM leads to integrin clustering and activation at the membrane, generating increased phoshatidylinositol lipids at adhesion sites. Rgnef PH domain associates with concentrated membrane lipids and facilitates FAK localization at nascent adhesions. FAK activation promotes FA maturation and Rgnef RhoA-GEF catalytic activity promotes actomyosin contractility, both required for proper cell motility.

Table 1. Known Human Dbl Family RhoGEFs and their Roles in Development

Dbl Protein	Other Names	Acc #	aa #	Target GTPases	Defect	References
α-Pix	ARHGEF6; Cool-2	Q15052	776	Rac1, Cdc42	Viable and fertile; deficient immune response, lower mature lymphocyte population, impaired spatial and complex learning	[98, 99]
β-Pix	ARHGEF7; Cool-1	Q14155	803	Rac1, Cdc42, Lrrk2	Embryonic lethal	[98, 100]
Abr		Q12979	859	RhoA, Rac1, Cdc42	Viable and fertile; cerebellar and vestibular defects with combined Bcr loss	[101, 102]
AKAP13	ARHGEF13; Lbc	Q12802	2813	RhoA	Early embryonic lethality with heart development defects	[50]
ALS2	Alsin	Q96Q42	1657	Rac1, Rab5	Viable and fertile; hypoactive behavior, shorter lifespan in some genetic backgrounds	[103, 104]
ARHGEF4	XPLN; STA3	Q9NR81	526	RhoA/B	In zebrafish: cytopenia, abnormal vascular development	[105, 106]
ARHGEF10	RhoGEF10	O15013	1369	RhoA	*unknown*	[107]
ARHGEF10L	GrinchGEF	Q9HCE6	1279	RhoA/B/C	*unknown*	[108]
ARHGEF16	Ephexin-4	Q5VV41	709	RhoG, Cdc42	*unknown*	[109, 110]
ARHGEF33	FLJ41381	A8MVXO	844	*unknown*	*unknown*	
ARHGEF37	FLJ41603	A1IGU5	675	*unknown*	*unknown*	
ARHGEF38	FLJ20184	Q9NXL2	219	*unknown*	*unknown*	
Asef1	ARHGEF4; ASEF	Q9NR80	690	Rac1, Cdc42	Viable and fertile; impaired retinal angiogenesis	[111]
Asef2	ARHGEF29; SPATA13	Q96N96	652	RhoA, Rac1, Cdc42	Viable and fertile	[112, 113]
BCR		P11274	1271	Rac1, Cdc42	Viable and fertile; increase in neutrophil respiratory burst	[114]
C9orf100	ARHGEF39; FLJ14642	Q8N4T4	335	*unknown*	*unknown*	[115]
Dbl	ARHGEF21; MCF2	P10911	925	RhoA/B/C/G, Rac1, Cdc42	Viable and fertile; dendrite elongation defect	[116]
Dbs	ARHGEF14; MCF2L	O15068	1137	RhoA, Rac1, Cdc42	Viable and fertile; lower B cell count and cholesterol, increased grip strength, hyperphosphatemia (males only)	[117, 118]
DNMBP	ARHGEF36; TUBA	Q6XZF7	1577	Cdc42	*unknown*	[119]
Ect2	ARHGEF31	Q9H8V3	914	RhoA/B, Rac1, Cdc42	Peri-implantation lethality	[52, 120]
Ect2L	ARHGEF32	Q008S8	904	*unknown*	*unknown*	
Ephexin-1	ARHGEF27; WGEF	Q8N5V2	710	RhoA, Rac1, Cdc42	Viable and fertile; severe muscle weakness in adults	[121]
FARP1	CDEP	Q9Y4F1	1045	RhoA, Rac1	*unknown*	[122, 123]
FARP2	FIR, FRG	O94887	1054	Rac1, Cdc42	Viable	[124-126]
FGD1	FGDY; ZFYVE3	P98174	961	Cdc42	Human genomic deletions cause Aarskog-Scott syndrome	[127]
FGD2	ZFYVE4	Q7Z6J4	655	Cdc42	*unknown*	[128]
FGD3	ZFYVE5	Q5JSP0	725	Cdc42	*unknown*	[129]

(Table 1) contd…..

Dbl Protein	Other Names	Acc #	aa #	Target GTPases	Defect	References
FGD4	CMT4H; Frabin; ZFYVE5	Q96M96	766	Cdc42	Viable and fertile; myelin abnormalities	[130]
FGD5	ZFYVE23	Q6ZNL6	1462	Cdc42	*unknown*	[131]
FGD6	ZFYVE24	Q6ZV73	1430	*unknown*	*unknown*	
GEF-H1	ARHGEF2; Lfc	Q92974	986	RhoA, RhoB	*unknown*	[132, 133]
hPEM-2	ARHGEF9; Collybistin	O43307	516	Cdc42	Loss of function in humans causes mental retardation and epilepsy	[134, 135]
Intersectin-1	ITSN1	Q15811	1721	Cdc42	Some early postnatal fatality; fertile, dysregulated neuronal vesicle trafficking	[136, 137]
Intersectin-2	ITSN2	Q9NZM3	1697	Cdc42	*unknown*	[138]
Kalirin	ARHGEF24; Duet, Duo	O60229	2985	Rac1	Viable and fertile; reduced cortex and hippocampal size, locomotor hyperactivity, memory impairment, abnormal social behavior	[139]
LARG	ARHGEF12	Q9NZN5	1544	RhoA	Viable and fertile; smooth muscle hypertension defects	[140]
MCF2L2	ARHGEF22	Q86YR7	1114	*unknown*	*unknown*	
MyoGEF	PLEKHG6	Q3KR16	790	RhoA/C/G, Rac1	*unknown*	[141, 142]
NET1	ARHGEF8	Q7Z628	596	RhoA/B/C	*unknown*	[120, 143, 144]
Obscurin	ARHGEF30; OBSCN	Q5VST9	7968	RhoA/Q	Viable and fertile; muscle weakness, mild age-dependent muscular myopathy	[145-148]
P-Rex1		Q8TCU6	1659	Rac1/2	Viable and fertile; reduced lung permeability, platelet secretion and aggregation, and neutrophil recruitment	[149-152]
P-Rex2		Q70Z35	1606	Rac1	Viable and fertile; altered Purkinje cell morphology, impaired motor coordination	[153, 154]
p114RhoGEF	ARHGEF18	Q6ZSZ5	1173	RhoA, Rac1	*unknown*	[155]
p115RhoGEF	ARHGEF1; LSC	Q6NX52	948	RhoA	Viable and fertile; leukocyte homeostasis defects, gastrointestinal motor dysfunctions	[56, 57, 156]
p164-RhoGEF	ARHGEF17; TEM4	Q96PE2	2063	RhoA/B/C	*unknown*	[157, 158]
p63RhoGEF	ARHGEF25; GEFT	Q86VW2	580	RhoA	*unknown*	[159]
PDZ-RhoGEF	ARHGEF11; PRG	O15085	1522	RhoA	Viable and fertile	[156, 160]
PLEKHG1	ARHGEF41	Q9ULL1	1385	*unknown*	Decreased granulocytes, decreased susceptibility to bacterial infection	
PLEKHG2	ARHGEF42; FLJ00018	Q9H7P9	1386	Rac1, Cdc42	*unknown*	[161]
PLEKHG3	ARHGEF43	A1L390	1219	*unknown*	Deleted in some human autism cases, learning difficulties	[162, 163]
PLEKHG4	ARHGEF44; SCA4	Q58EX7	1191	*unknown*	Human genetic mutations associated with spinocerebellar ataxia	[164]
PLEKHG4B	KIAA1909	Q96PX9	1271	*unknown*	*unknown*	
PLEKHG5	DSMA4; GEF720	O94827	1062	RhoA	Human genetic mutations associated with distal spinal muscular atrophy	[165, 166]

(Table 1) contd…..

Dbl Protein	Other Names	Acc #	aa #	Target GTPases	Defect	References
PLEKHG7		Q6ZR37	379	*unknown*	*unknown*	
RasGRF1	CDC25; GRF1	Q13972	1275	Ras, Rac1	Viable and fertile; reduced body weight and impaired growth, glucose homeostasis and retinal defects, impaired long-term memory, longer lifespan	[167-172]
RasGRF2	GRF2	O14827	1237	Ras, Rac1	Viable and fertile; impaired T cell signaling	[173, 174]
Rgnef	ARHGEF28; p190RhoGEF	Q8N1W1	1705	RhoA/C	Partial embryonic lethality; fertile, decreased size at birth	[22, 44]
SGEF	ARHGEF26	Q96DR7	871	RhoG	Viable and fertile	[175, 176]
Solo	ARHGEF40; Scambio	Q8TER5	1519	RhoA/C	*unknown*	[177]
SOS1	GF1	Q07889	1333	Ras, Rac1	Embryonic lethal	[51, 178, 179]
SOS2		Q07890	1332	Ras, Rac1	Viable and fertile	[179, 180]
Tiam1		Q13009	1591	Rac1, Cdc42, RhoA	Partial embryonic lethality; fertile, smaller brain size, some anencephaly and exencephaly	[181, 182]
Tiam2	STEF	Q8IVF5	1701	Rac1	*unknown*	[183]
TIM-1	ARHGEF5; Ephexin-3	Q12774	1597	RhoA/B/C/G	Viable and fertile; decrease in dendritic cell migration	[184]
Trio	ARHGEF23	O75962	3038	RhoA/G, Rac1	Embryonic lethal; muscle and neural tissue defects	[53]
VAV1	VAV	P15498	845	RhoA/G, Rac1, Cdc42	Viable and fertile; T cell development defects	[185, 186]
VAV2		P52735	878	RhoA/G, Rac1, Cdc42	Viable and fertile; cardiovascular remodeling, renal dysfunction	[187, 188]
VAV3		Q9UKW4	847	RhoA/G, Rac1, Cdc42	Viable and fertile; large bones, cardiovascular remodeling, tachycardia, hypertension, renal dysfunction, cerebellar defects	[189, 190]
Vsm-RhoGEF	ARGEF15; Ephexin-5	O94989	841	Cdc42	Viable and fertile; reduced retinal vasculature growth	[191]
WGEF	ARHGEF19; Ephexin-2	Q8IW93	802	RhoA, Cdc42, Rac1	*unknown*	[192]

Acc #, human protein accession number; aa #, protein amino acid length; Defects as determined by human pathology or targeting appropriate GEF homolog in other animal species.

embryonic lethal phenotypes are uncommon in other RhoGEF transgenic mouse models (Table **1**). Except for AKAP13 (ARHGEF13) [50], Sos1 [51], Ect2 (ARHGEF31) [52], β -Pix (ARHGEF7), and Trio (ARHGEF23) knockouts which result in embryonic lethality [53], other RhoGEF knockouts have non-lethal phenotypes (Table **1**).

Interestingly, as observed with loss of Rgnef, knockout of the RhoA effector proteins ROCK1 or ROCK2 (Rho-associated protein kinases) also result in partial embryo lethality and birth of small pups [54, 55]. ROCK2 loss was associated with late placental dysfunction and ROCK1 loss with cellular actomyosin bundling defects. Future studies of Rgnef knockout embryos in utero will be focused on identifying potential phenotypes as a means to link Rgnef to RhoA signaling *in vivo*. Many of the restricted hematopoietic or neural defects associated with RhoGEF loss are linked to potential alterations in cell movement (Table **1**). For instance, Lsc/p115 (ARHGEF1) loss is associated with marginal zone B-cell and neutrophil migration defects [56, 57]. In culture, Rgnef-/- fibroblasts exhibit defects in adhesion formation and cell movement when stimulated by extracellular matrix proteins such as fibronectin [44]. This has been associated with decreased integrin-mediated signaling to RhoA as well as FAK activation as discussed below.

INTEGRIN-RHOA SIGNALING AXIS

Integrin receptors are heterodimeric transmembrane proteins comprised of alpha and beta subunits that cluster upon binding to extracellular matrix proteins and signal across the membrane in both directions [58]. Integrins generate signals within cells with respect to

external surroundings and establish a physical linkage to the actin cytoskeleton to facilitate cell adhesion, shape change, and tension. Cell adhesion complexes (also called focal adhesions, FAs) consist of integrins and various cytoplasmic proteins such as talin, vinculin, paxillin, and alpha-actinin. FA formation is associated with the activation of kinases, including FAK and c-Src, that phosphorylate substrates such as p130Cas or cortactin promoting the binding of adaptor proteins like Crk or Nck and the establishment of large multi-protein signaling complexes at FAs. Linkages of Crk and Nck to actin nucleating protein complexes such as N-WASP or Arp2/3 alter actin branching with effects on cell protrusion activity. These early signaling events are associated with cell spreading, cycles of GTPase activation and inactivation, which occur concurrent with the formation, maturation, and eventual turnover of FAs [59]. All of these events must be precisely coordinated to enable efficient directional cell movement.

Canonical cell migration models postulate that Rac promotes membrane protrusion at the leading edge and Rho regulates contractility in the cell body [7]. However, studies with FRET-based probes for Rho GTPases revealed high levels of RhoA activity at both the leading and trailing edges of cells [60]. The occurrence of high Rac and Rho activity at leading edge is likely cyclical and/or may occur at distinct sites. At the leading edge, Rac activation can provide the necessary “push” (decrease in cell contractility) needed for lamellipodial growth and Rho activation then facilitates the “pull” (increase in cell contractility) to stabilize growing lamellipodia in part through FA maturation [61].

Biochemically, cell adhesion to fibronectin (FN) initially triggers an overall transient decrease in RhoA activity levels (at 15 to 30 min), followed by an extended phase of RhoA activation associated with FA maturation [62, 63]. It is the coordination of GAP and GEF activity that promotes RhoA cyclic regulation upon FN binding. Interestingly, FAK is linked to FN-mediated cyclic RhoA regulation through associations with both p190RhoGAP [64] and Rgnef [29]. FAK expression and activity promoted FA localization and tyrosine phosphorylation of p190RhoGAP [34, 64] and this is associated with increased GAP activity, cell protrusion, and establishment of polarity [65]. The FAK-p190RhoGAP interaction is indirect and dependent upon the binding of p120RasGAP to both FAK and p190RhoGAP [64]. In the absence of FAK expression or activity, RhoA activity is high and deregulated [62]. In addition to the loss of p190RhoGAP regulation, FAK-/- fibroblasts exhibit high levels of Rgnef expression due in part to compensatory signaling from the FAK-related Pyk2 kinase [29]. Elevated Rgnef expression contributes to aberrant FAK-/- fibroblast morphology, RhoA activity, and increased FA formation. However, in normal fibroblasts, Rgnef knockdown prevents FN-stimulated RhoA regulation, FA formation, and cell motility [29]. Despite published putative roles for LARG (ARHGEF12), Lsc/p115 (ARHGEF1), and GEFH1 (ARHGEF2) in FN-stimulated RhoA regulation [66, 67], Rgnef knockout fibroblasts exhibit defects in FN-stimulated RhoA regulation that are rescued by Rgnef re-expression [44]. Taken together, these studies establish the importance of Rgnef in RhoA regulation downstream of integrins. Simplistically, too much or not enough Rgnef expression in cells inhibits cell movement, as the formation of overabundance or too few FAs limits cell motility.

COMPLEX INTERACTIONS BETWEEN RGNEF AND FAK

In this integrin-Rho signaling axis, it remains undetermined how Rgnef becomes activated to facilitate RhoA GTP binding. Using a binding assay with a nucleotide-free mutant of RhoA [66], Rgnef became activated 60 min after replating cells on FN [44]. Rgnef tyrosine phosphorylation after FN replating occurs at 60 min and this was disrupted by deletion of the FAK binding site (1292-1301) on Rgnef [29]. Rgnef tyrosine phosphorylation is associated with the localization of Rgnef to FAs and this is correlated with the ability of Rgnef to activate RhoA. However, the molecular mechanisms linking integrin signaling to Rgnef and RhoA activation is undetermined. In particular, it is not known how phosphorylation and the activity of different Rgnef domains act to control Rgnef function.

Despite over twenty years of research on FAK [68], the mechanisms through which FAK associates with integrin signaling complexes at FAs also remains unclear. Although FAK and paxillin co-localize to the earliest adhesions formed upon cell attachment to FN [69], other mutational and knockout studies have concluded that paxillin is important but not essential for FAK recruitment to nascent adhesions [70, 71]. Additionally, direct binding between FAK and talin may contribute to but is not essential for adhesion localization of FAK [72, 73]. It is the C-terminal region of FAK termed the focal adhesion targeting (FAT) domain that binds to paxillin and talin and facilitates FAK localization to integrin adhesion sites. The FAK FAT domain also binds to Rgnef residues 1292-1301 [28].

Interestingly, Rgnef Δ1292-1301 over-expression results in a similar phenotype to neurons that lack FAK [74]. This result was originally interpreted as Rgnef being downstream of FAK and that Δ1292-1301 Rgnef would block signaling leading to RhoA activation. However, an alternative possibility is that if Rgnef also functions upstream of FAK, expression of Rgnef Δ1292-1301 would not bind FAK and may inhibit FAK. To this end, recent studies in Rgnef-/- fibroblasts found that FAK activation (FAK Y397 phosphorylation) and paxillin tyrosine phosphorylation were inhibited at early time points (5 to 30 min) after cell adhesion to FN [38]. This was associated with decreased FAK co-localization at FAs. Rgnef mutagenesis and re-expression studies found that the Rgnef PH domain or FAK binding region were required as part of a mechanism promoting FAK FA localization, FAK activation, and paxillin tyrosine phosphorylation. Interestingly, Rgnef PH domain mutation (R1098A,

K1100A) prevented phosphatidylinositol 4-P and phosphatidylinositol 4,5P2 binding and these residues are conserved within related GEFs (Fig. **1B**). Modeling of the Rgnef DH-PH domain structure reveals that R1098 and K1100 may be located within a surface exposed pocket that could potentially form a phosphatidylinositol headgroup binding site (Fig. **1C**). In this way, it is likely that Rgnef lipid binding and scaffolding play an unexpected but important role in promoting FAK recruitment and activation at FAs.

Moreover, re-expression of a GEF-inactivating Rgnef point mutation (Y1003A) [21] in Rgnef-/- fibroblasts was sufficient to promote FAK FA localization and activation upon cell adhesion to FN [38]. However, Rgnef Y1003A did not promote paxillin tyrosine phosphorylation. This separates FAK and paxillin tyrosine phosphorylation downstream of integrins. Interestingly, myosin II activity and the generation of cell tension promote FAK-mediated paxillin tyrosine phosphorylation leading to adhesion maturation and cytoskeletal-matrix linkage reinforcement [75]. Thus, since Rgnef-/- fibroblasts do not efficiently activate RhoA upon cell adhesion to FN [44], and RhoA activation of ROCK can stimulate cell tensional forces through myosin-mediated contractility [76], it may be that Rgnef-mediated RhoA activation allows for FAK-mediated paxillin tyrosine phosphorylation at FAs in response to contractility signals or FA maturation.

As summarized in a simplistic model (Fig. **1D**), cell binding to matrix leads to integrin receptor clustering and activation. Signals are generated to increase phosphatidylinositol lipids within the plasma membrane near adhesion sites, and this facilitates Rgnef membrane association *via* the Rgnef PH domain. FAK binding to Rgnef is not regulated by cell adhesion, but the translocation of Rgnef to the membrane brings FAK to nascent adhesion sites and likely facilitates the formation of a complex between FAK and paxillin within FAs. Through processes that remain unclear, but may involve release of intramolecular inhibitory constraints [77] and intermolecular FAK transphosphorylation at Y397 [78], FAK becomes catalytically active. Rgnef-mediated RhoA activation and increased contractility facilitate FAK-mediated paxillin tyrosine phosphorylation important for FA maturation and the further recruitment of proteins such as vinculin to FAs. Inhibition of any of these steps prevents efficient cell movement.

RGNEF AND RHO - MORE THAN ONE CONNECTION

The recombinant DH-PH domain of Rgnef possesses exchange activity for RhoA and this is blocked by a point mutation (Y1003A) within the DH domain [21]. It is the DH domain that provides the canonical interface for Rho GTPase binding. PH domains bind to lipids and other protein targets [17]. Mutagenesis and *in vitro* binding assays have confirmed that the Rgnef PH domain binds phosphatidylinositol lipids and this is mediated in part by Rgnef residues R1098A and K1100A [38]. The PH domain of Rgnef also bound directly to activated RhoA and this was dependent on hydrophobic residues F1154 and I1156 [79]. In three-dimensional models of the Rgnef PH domain, this hydrophobic patch does not overlap with the R1098A and K1100A residues involved in phosphatidylinositol lipid binding (Fig. **1C**). Interestingly, mutation of Rgnef F1154 and I1156 in the full-length protein also attenuated RhoA activation, as assayed by a serum-response element gene reporter, when compared to wild type Rgnef [79]. This RhoGEF-activated RhoA binding interaction is conserved within the Lbc-family of RhoGEFs. It is proposed that this interaction could serve as a positive feedback loop, perhaps working in tandem with PH domain lipid-binding residues to correctly orient RhoGEFs at the plasma membrane or relieving auto-inhibition. In fact, several unrelated proteins including RhoGEFs have been shown to bind to activated GTPases through their PH domain, suggesting that this could be a common regulatory mechanism [80-82]. It will be of interest to test whether this Rgnef hydrophobic patch regulates its subcellular localization and whether the Rgnef PH domain also binds efficiently to other GTPases such as RhoC. This adds another layer to the possible mechanisms by which RhoA and RhoC are spatiotemporally regulated in normal and transformed cells.

RGNEF AND FAK IN CANCER

Studies of the molecular mechanisms controlling FAK activation are of potential clinical importance due to the fact that FAK controls various aspects of tumor progression [83]. Small molecules that act as ATP-competitive inhibitors of FAK activity are in various stages of development and human clinical trial testing [84-88]. What remains unclear are the molecular mechanisms driving elevated FAK activation in tumor cells. Notably, Rgnef mRNA and protein expression are significantly increased during colorectal tumor progression and dominant-negative expression of the Rgnef C-terminal domain resulted in smaller, less invasive tumors with reduced paxillin tyrosine phosphorylation as analyzed in an orthotopic model [37]. This tumor inhibitory activity of Rgnef-C required the presence of the FAK binding site and we speculate it may be associated with the prevention of FAK or Rho GTPase activation. Early studies identified Dbl (ARHGEF21) in a cell transformation-based screen [89], various RhoGEFs are over-expressed in tumors [90], and small molecule inhibitors of RhoGEFs that disrupt binding to RhoGTPases are being developed [91]. Thus, targeted inhibition of RhoGEFs like Rgnef may result in dual inhibition of FAK and Rho GTPase signaling pathways.

Mechanistic screens for RhoGEF inhibitors include *in vitro* invasion assays, as RhoA and RhoC GTPases have been linked to an invasive cell phenotype [76]. In fact, recent studies point to the importance of a RhoA-FAK signaling axis in KRAS-driven non-small cell lung cancer (NSCLC) [92]. This study concluded that since RhoA silencing and FAK pharmaceutical inhibition yielded similar anti-tumor effects on NSCLC tumor

bearing KRAS and INK4A/Arf mutations, that activation of a RhoA-FAK signaling axis is a genotype-specific vulnerability of high grade tumors. FAK activity is also an important factor promoting breast cancer tumor growth and metastasis [87, 93, 94]. Structures termed invadopodia on carcinoma cells degrade surrounding matrix and allow for enhanced tumor cell invasion [95]. In breast carcinoma cells, Rgnef was shown to activate RhoC to facilitate invadopodia formation [22]. Although functional connections between FAK and Rgnef have not been established in breast cancer, invasive matrix degradation is dependent upon FAK signaling [93, 96]. Understanding the mechanisms of Rgnef spatiotemporal regulation and interactions with FAK and RhoA or RhoC GTPases *in vivo* will provide new insights on the molecular pathways involved in cancer progression.

CONCLUDING REMARKS

In this review we have emphasized the dual function of Rgnef, which acts as a GEF for RhoA and RhoC, and plays a novel scaffolding role in FAK recruitment and activation. The Rgnef-FAK interaction is critical for both normal cell migration and tumorigenesis, as FAK contributes to several hallmarks of cancer, including survival, proliferation, angiogenesis, and invasion. Future studies will be aimed at understanding the molecular mechanisms behind Rgnef-FAK signaling in tumor progression to better understand how these pathways can be targeted in the future for more effective treatments.

Further, the recent discovery of novel RhoA-GTP binding patch on the PH domain provides a new opportunity to understand how Rgnef spatiotemporally regulates Rho GTPases, and vice versa. Due to recent evidence that a lipid-binding mutation in the PH domain prevents FAK membrane localization, further investigation of the role of the Rgnef PH domain with regard to lipid binding, necessity in promoting FAK activation, and interactions with RhoA/C in the context of tumor progression are warranted.

The use of Rgnef-null mouse and cell lines has provided a powerful system to dissect signaling pathways downstream of integrins at focal adhesions. Already, the use of these cells has revealed a novel method of FAK recruitment and allowed us to separate FAK and paxillin phosphorylation downstream of integrins for the first time. These knockout systems will be a valuable tool in examining the role of Rgnef and its binding partners in cellular signaling, development, and cancer.

ABBREVIATIONS

ATP = Adenosine triphosphate
C-terminus = Carboxy terminus
Dbl = Diffuse B-cell lymphoma
DH = Dbl-homology
ECM = Extracellular matrix
FA = Focal adhesion
FAK = Focal adhesion kinase
FAT = Focal adhesion kinase
FN = Fibronectin
FRET = Fluorescence resonance energy transfer
GAP = GTPase activating protein
GDP = Guanosine diphosphate
GTP = Guanosine triphosphate
NSCLC = Non-small cell lung cancer
PDZ = Post synaptic density protein, disc large tumor suppressor, zona occludens-1
PH = Pleckstrin homology
ROCK = Rho-associated protein kinase

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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