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REVIEW

G protein signaling in the parasite *Entamoeba histolytica*

Dustin E Bosch[1] and David P Siderovski[2]

The parasite *Entamoeba histolytica* causes amebic colitis and systemic amebiasis. Among the known amebic factors contributing to pathogenesis are signaling pathways involving heterotrimeric and Ras superfamily G proteins. Here, we review the current knowledge of the roles of heterotrimeric G protein subunits, Ras, Rho and Rab GTPase families in *E. histolytica* pathogenesis, as well as of their downstream signaling effectors and nucleotide cycle regulators. Heterotrimeric G protein signaling likely modulates amebic motility and attachment to and killing of host cells, in part through activation of an RGS-RhoGEF (regulator of G protein signaling–Rho guanine nucleotide exchange factor) effector. Rho family GTPases, as well as RhoGEFs and Rho effectors (formins and p21-activated kinases) regulate the dynamic actin cytoskeleton of *E. histolytica* and associated pathogenesis-related cellular processes, such as migration, invasion, phagocytosis and evasion of the host immune response by surface receptor capping. A remarkably large family of 91 Rab GTPases has multiple roles in a complex amebic vesicular trafficking system required for phagocytosis and pinocytosis and secretion of known virulence factors, such as amebapores and cysteine proteases. Although much remains to be discovered, recent studies of G protein signaling in *E. histolytica* have enhanced our understanding of parasitic pathogenesis and have also highlighted possible targets for pharmacological manipulation.
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ENTAMOEBA HISTOLYTICA CAUSES AMEBIC COLITIS AND SYSTEMIC AMEBIASIS

Epidemiology, disease sequelae and current treatment options

The parasite *E. histolytica* is the causative agent of infectious amebic colitis and systemic amebiasis.[1] The worldwide prevalence of *E. histolytica* infection is not precisely known, with the most recent published estimates[2] being approximately 50 million infections and 100 000 deaths annually. Epidemiological estimates have been historically complicated by limitations of diagnostic tests, as well as difficulty in differentiating *E. histolytica* from the morphologically similar but typically non-pathogenic related *Entamoeba* species, *E. dispar* and *E. moshkovskii*.[3] However, more recently developed antigen detection and PCR-based modalities with improved sensitivity and specificity have allowed more accurate regional estimations of *E. histolytica* infections.[4,5] The prevalence of *E. histolytica* infection is particularly high among susceptible populations with limited access to clean water. For instance, a study of preschool-aged children in Bangladesh revealed annual infections in 40–50% of subjects,[6] a profile of Orang Asli ethnic groups in Malaysia found an overall prevalence of *E. histolytica*-positive stool samples to be 15–20%,[7] and *E. histolytica* was detected by PCR in 10–15% of a rural Mexican population.[8] The prevalence of antibodies specific for *E. histolytica* in sera of a Chinese population varied from 0.5 to 14%, depending on geographical location.[9] An inter-relationship between host nutritional status and susceptibility to *E. histolytica* infection has also recently begun to emerge (reviewed in Verkerke *et al.*[10]). Although *E. histolytica* infection is relatively rare in developed countries, such as the United States, it does occur among travelers, immigrants and select susceptible subpopulations.[11,12] Furthermore, outbreaks of *E. histolytica* have occurred due to contaminated municipal water supplies (for example, Barwick *et al.*[13]).

The life cycle of *E. histolytica* consists of an interchange between an encysted form and a motile, pathogenic,

[1]Department of Pharmacology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA and [2]Department of Physiology and Pharmacology, West Virginia University School of Medicine, Robert C. Byrd Health Sciences Center, Morgantown, WV, USA
Correspondence: Dr DP Siderovski, Department of Physiology and Pharmacology, 3051A Health Sciences Center, West Virginia University School of Medicine, Morgantown, WV 26506-9229, USA.
Email: dpsiderovski@hsc.wvu.edu
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trophozoite form. *E. histolytica* cysts, shed in the feces of infected human hosts, are transmitted primarily by ingestion of contaminated water or food.[1] Excystation occurs in the small intestine, and the resultant *E. histolytica* trophozoites may then colonize the large intestine while evading the host immune response.[3] Although the majority of *E. histolytica* infections are asymptomatic, trophozoites can penetrate the intestinal mucous barrier, resulting in colitis.[1] Amebic colitis is characterized by trophozoite-mediated killing of intestinal epithelial cells and responding immune cells, as well as local tissue destruction.[14] In rare cases, *E. histolytica* trophozoites can enter the blood stream and spread systemically, giving rise to abscesses, primarily in the liver and less frequently in the lungs and brain.[3] Although systemic amebiasis requires previous intestinal infection, amebic liver abscesses can develop in the absence of symptomatic colitis[14,15] and are known to appear months or years following exposure.[16] Thus, treatment is recommended for patients with *E. histolytica* infection, even in the absence of symptomatic disease.[3]

Nitroimidazoles, such as metronidazole, are the current best drugs for the treatment of invasive amebiasis.[3] Approximately 90% of patients with mild or moderate amebic colitis respond to nitroimidazole therapy, although persistent intestinal infection often requires additional treatment with paromomycin or diloxanide furoate for complete eradication.[3] However, a significant fraction of patients with *E. histolytica* infection do not respond to nitroimidazoles, and relatively rare side effects such as allergic reactions, neuropathies and additional gastrointestinal symptoms can also affect treatment tolerance.[17] Resistance of *E. histolytica* infection to nitroimidazoles and paromomycin has not yet emerged as a major limitation to treatment; however, numerous examples of antibiotic resistance in other microorganisms warrants further exploration of alternative pharmacological therapeutics.[18] A recent study identified auranofin, an FDA (Food and Drug Administration)-approved rheumatoid arthritis drug, as a potent inhibitor of *E. histolytica* thioredoxin reductase and further demonstrated its protective effects in a mouse model of amebic colitis.[19] Other classes of compounds have also recently been pursued as nanomolar-potency inhibitors of *E. histolytica* growth in culture.[20,21] Despite existing effective therapies, *E. histolytica* infection and associated disease remains endemic in many parts of the world, particularly in areas with contaminated drinking water and food sources.[6,8] Problems with sanitation implementation and access to appropriate therapeutics could potentially be circumvented by the development of an *E. histolytica* vaccine, and efforts toward this goal are ongoing (for example, Abd Alla *et al.*[22]).

Parasite factors in pathogenesis

A number of *E. histolytica* molecular components have been thoroughly established as contributors to its pathogenesis. During initial intestinal colonization, *E. histolytica* adheres to the colonic mucin layer primarily through a galactose-inhibitable lectin, known as the Gal/GalNAc lectin (reviewed in Petri *et al.*[23]). The trimeric surface protein is also a dominant factor in parasite attachment to host cells and subsequent tissue destruction and functions interdependently with the dynamic actin cytoskeleton of *E. histolytica*.[23] Trophozoites also secrete pore-forming peptides known as 'amebapores' that assemble within host cell membranes to trigger cell death (reviewed in Leippe and Herbst[24]). A relatively large family of *E. histolytica*-encoded cysteine proteases also contributes to host cell killing, as well as degradation of the host extracellular matrix during invasive amebic infection and evasion of the host immune response through proteolysis of immunoglobulins and complement (reviewed in Que and Reed[25]). Many regulators of the actin-rich cytoskeleton within *E. histolytica* are also emerging as contributors to pathogenesis-related processes, such as phagocytosis of host cells, trophozoite motility and tissue invasion, and shedding of host antibodies by surface receptor capping (reviewed in Voight and Guillen,[26] Tavares *et al.*,[27] Meza *et al.*[28] and Labruyere and Guillen[29]).

HETEROTRIMERIC G PROTEINS AND RAS SUPERFAMILY GTPASES

Sequencing of the complete *E. histolytica* genome[30] and genome-wide expression studies (for example, Gilchrist *et al.*[31]) have revealed large numbers of putative cell signaling molecules expressed in this single-celled parasite, including a substantial family of >300 kinases.[32] Also prominent within the *E. histolytica* genome are genes encoding heterotrimeric G protein subunits (Gα, Gβ and Gγ) and a large number of small, $\sim$21 kDa G proteins belonging to the Ras superfamily.[30] Gα subunits and Ras GTPases are molecular switches and cellular signaling nodes that bind guanine nucleotides (guanosine triphosphate (GTP) or guanosine diphosphate (GDP)) through highly conserved, nucleotide-interacting sequencing motifs.[33,34] As mammalian G proteins are known to be master regulators of cellular functions spanning cell division and proliferation, cytoskeletal dynamics, vesicular trafficking and specific responses to extracellular cues,[33,35] it is likely that *E. histolytica* homologs are similarly important for trophozoite biology and pathogenicity. G protein signaling pathways are also notable for their amenability to pharmacological manipulation; particularly, heterotrimeric G protein signaling *via* G protein-coupled receptors (GPCRs) is the target of approximately one-third of all currently FDA-approved drugs.[36,37]

REGULATION OF THE GUANINE NUCLEOTIDE CYCLE

Heterotrimeric G proteins

A Gα subunit in its inactive, GDP-bound state forms a heterotrimer with the obligate Gβγ dimer (Figure 1). A seven-transmembrane domain GPCR, when activated by an extracellular ligand, engages the heterotrimer and catalyzes the release of GDP by the Gα subunit.[38] Thus the GPCR is a guanine nucleotide exchange factor (GEF) for the Gα subunit, promoting GDP release and subsequent binding of GTP, which is present in a higher concentration than GDP in the

cytoplasm.[34] Nucleotide exchange is accompanied by structural rearrangement of three switch regions in the Ras-like domain of the Gα subunit, resulting primarily from nucleotide-binding pocket interactions with the γ-phosphoryl group of GTP.[39] The activated Gα · GTP separates from the Gβγ dimer, and both components are free to signal through various downstream effectors.[34] Mammalian Gα subtypes engage different effectors: $G\alpha_s$ activates, while $G\alpha_{i/o}$ inhibits, cyclic AMP (cAMP) generation by adenylyl cyclase; $G\alpha_q$ stimulates phospholipase-Cβ activity and subsequent release of intracellular calcium stores; and $G\alpha_{12/13}$ signaling leads to Rho GTPase activation through RhoGEFs.[34,40] Signaling is terminated by the intrinsic GTPase activity of the Gα subunit, leading to release of free phosphate and repeated formation of the Gα · GDP/βγ heterotrimer (Figure 1). Gα subunit-mediated GTP hydrolysis, and thus signal termination, is accelerated by a family of GTPase-accelerating proteins (GAPs) known as 'regulators of G protein signaling' (RGS proteins).[41] RGS proteins do not directly contribute to the GTP hydrolysis reaction, but instead stabilize the Gα switch regions to allow for efficient hydrolysis.[42] Some Gα subunit effectors also enhance GTPase activity; particularly, phospholipase-Cβ serves as a GAP for $G\alpha_q$, and the $G\alpha_{12/13}$ subfamily RGS-RhoGEF effectors possess a GTPase-accelerating domain (the rgRGS domain) with distant homology to RGS proteins.[40,43] An additional class of Gα regulators is the GoLoco motif protein family, members of which serve as guanine nucleotide dissociation inhibitors (GDIs) by binding directly to Gα · GDP and preventing nucleotide release.[44]

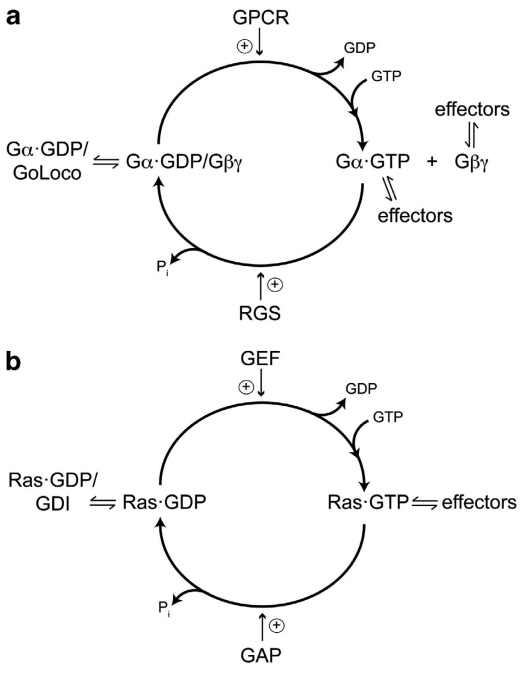

Figure 1 Nucleotide cycle regulation of heterotrimeric and Ras superfamily G proteins. (**a**) Gα subunits cycle between guanosine diphosphate (GDP)- and guanosine triphosphate (GTP)-bound states. G protein-coupled receptors (GPCRs) serve as guanine nucleotide exchange factors (GEFs) for G protein heterotrimers, stimulating their release of GDP. Conversely, GoLoco motifs are guanine nucleotide dissociation inhibitors (GDIs) that slow nucleotide exchange by Gα subunits. RGS proteins are GTPase-accelerating proteins (GAPs) for Gα subunits, promoting signal termination by both activated Gα subunits and free Gβγ. (**b**) The small G protein nucleotide cycle parallels that of heterotrimeric G proteins, with GEF-stimulated and GDI-inhibited nucleotide exchange as well as GAP-mediated activation of GTP hydrolysis. RGS, regulators of G protein signaling.

Ras superfamily GTPases

The nucleotide cycle of Ras superfamily G proteins closely parallels that of heterotrimeric G proteins. Inactive, GDP-bound Ras GTPases are activated by guanine nucleotide exchange factors (GEFs) in a process that involves structural rearrangement of two switch regions within the G protein to promote release of GDP and the Mg^{2+} cofactor (Figure 1).[33,45] Following binding of GTP, activated Ras superfamily GTPases engage a host of different downstream effectors. In contrast to heterotrimeric G proteins, the intrinsic GTPase activity of Ras superfamily members is typically very slow. Thus, Ras superfamily-specific GAPs truly 'activate' GTP hydrolysis (rather than merely accelerate hydrolysis as is the case with Gα GAPs) by contributing directly to the reaction, as typified by the 'arginine finger' of p120GAP.[46,47] In another distinct difference with Gα subunits, Ras superfamily GTPases typically possess a C-terminal cysteine residue that is isoprenylated in cells by specific lipid moiety transferases, a post-translational modification that promotes their membrane association.[33] GDIs associated with Ras superfamily GTPases slow nucleotide exchange and employ an isoprenyl group-binding site to extract GTPases from, and shuttle them between, cellular membranes.[48,49]

HETEROTRIMERIC G PROTEIN SIGNALING IN *E. HISTOLYTICA*

Before completion of the *E. histolytica* genome sequencing project,[30] indirect evidence for heterotrimeric G protein signaling components existing within *E. histolytica* accumulated in the literature, but specific genes and associated protein products had not been identified. Studies on the effects of histamine and serotonin, typical GPCR agonists, on *E. histolytica* trophozoites revealed alterations in pathogenicity and phagocytic activity, as well as enhancement of virulence in a mouse model,[50–53] suggesting the possible presence of a hormone-sensing G protein signaling pathway within *E. histolytica*. Exposure of *E. histolytica* to fibronectin fragments was seen to result in actin cytoskeleton rearrangements, as well as changes in intracellular calcium and cAMP levels,[54–56] raising the possibility of fibronectin-responsive $G\alpha_q$, $G\alpha_s$ and/or $G\alpha_{i/o}$ signaling within trophozoites. Additional indirect evidence arose from studies utilizing cholera toxin (CTX) and pertussis toxin (PTX), factors known to adenosine diphosphate (ADP)-ribosylate and activate $G\alpha_s$ or inhibit $G\alpha_{i/o}$ signaling, respectively.[57] Both CTX and PTX were reported to ADP-ribosylate

multiple proteins of diverse molecular weights in trophozoite lysates, and toxin treatment led to increased cAMP formation in both cytoplasmic and cell membrane preparations, as well as increased adhesion to a fibronectin-coated surface.[58] Studies in the related species *Entamoeba invadens* further suggested the possibility of heterotrimeric G protein signaling in *Entamoeba*. The catecholamines epinephrine and norepinephrine, classical GPCR agonists in mammals, were found to promote *E. invadens* encystation at high-nanomolar to low-micromolar concentrations, although a traditional concentration–response pattern was not observed.[59] The authors hypothesized the presence of a β1 adrenergic receptor-like molecular entity on trophozoite cell surfaces, as further supported by radioligand binding with a specific antagonist. Furthermore, chromatography techniques identified catecholamines within *E. histolytica* extracts, suggesting a potential autocrine G protein signaling loop.[59] Additional studies implied that CTX or PTX treatment, as well as the adenylyl cyclase-stimulating compound forskolin, could also promote cAMP accumulation in (and encystation of) *E. invadens*, while application of an adenylyl cyclase inhibitor was reported to have opposite effects.[60] Together with epinephrine-induced binding of GTPγS on trophozoite membranes, these findings were suggestive of an adrenergic receptor signal transduction cascade within *Entamoeba* involving $G\alpha_s$- and/or $G\alpha_{i/o}$-like proteins, with opposing regulatory effects on an adenylyl cyclase.

However, the sequenced *E. histolytica* genome,[30] as well as those of *E. dispar* and *E. invadens*, have revealed only the presence of two putative Gα subunits, a single Gβ subunit and at least two Gγ subunits.[61,62] Absent from the genome are clear homologs to mammalian phospholipase-Cβ as well as G protein-regulated adenylyl cyclases or cyclic nucleotide phosphodiesterases.[30] Thus, although exposure of *E. histolytica* to stimuli such as fibronectin and catecholamines may result in cAMP accumulation or increased intracellular calcium levels, it is unlikely that these observed effects are mediated by traditional $G\alpha_s$/adenylyl cyclase, $G\alpha_{i/o}$/adenylyl cyclase or $G\alpha_q$/phospholipase-Cβ signaling pathways. Also, we have been unable to identify within the *E. histolytica* genome any genes encoding clear homologs of adrenergic, histamine or serotonin GPCRs (unpublished data and Bosch *et al.*[61]), suggesting that the functional effects of these biogenic amines on trophozoites may not be mediated by traditional GPCR/heterotrimeric G protein signal transduction.

Analysis of both the sequence and structure of the Gα subunit EhGα1 revealed a lack of homology to mammalian Gα subfamilies, including $G\alpha_s$ and $G\alpha_{i/o}$.[61] This finding, together with a lack of the C-terminal cysteine required for ADP ribosylation by PTX, suggests that EhGα1 is unlikely to be specifically modified by bacterial toxins.[61] The observed effects of CTX and PTX treatment on *Entamoeba* trophozoites might instead result from off-target effects, a hypothesis supported by CTX- and PTX-mediated ADP ribosylation of multiple proteins of diverse molecular weights in *E. histolytica* trophozoite lysates.[58] Despite its lack of phylogenetic relationship to any particular mammalian Gα subfamily,[61] EhGα1 shares functional similarity with mammalian $G\alpha_{12/13}$ subunits in engaging and contributing to the activation of an RGS-RhoGEF effector (Figure 2).[63] An evolutionary origin of *E. histolytica* heterotrimeric G protein signaling independent from, but functionally convergent with, that of mammalian $G\alpha_{12/13}$/RGS-RhoGEF pathways is suggested by multiple factors, including the sequence and structural divergence of EhGα1, the canonical nature of its interaction with the EhRGS-RhoGEF RGS domain (that is, as opposed to the rgRGS domain found in mammalian RhoGEFs), and the structural features of the autoinhibited EhRGS-RhoGEF.[61,63] Expression of constitutively active EhGα1 and EhRacC mutants, together with the effector EhRGS-RhoGEF, leads to Rho family GTPase activation in *Drosophila* S2 cells,[63] suggesting that heterotrimeric G protein and Rho family GTPase signaling pathways communicate in *E. histolytica* (Figure 2). However, no specific Rho family GTPase has yet been identified as an EhRGS-RhoGEF substrate. Overexpression of either wild-type EhGα1 or a dominant-negative, constitutively EhGβγ-bound EhGα1 mutant has opposing effects on trophozoite migration, invasion, and host cell attachment and killing, suggesting that heterotrimeric G protein signaling modulates multiple pathogenesis-related behaviors.[61] Perturbation of EhGα1 expression also leads to significant changes in the *E. histolytica* transcriptome and alters the secretion of cytotoxic cysteine proteases,[61] suggesting a possible functional overlap with Rab family GTPases (see below). Overexpression of EhRGS-RhoGEF has similar

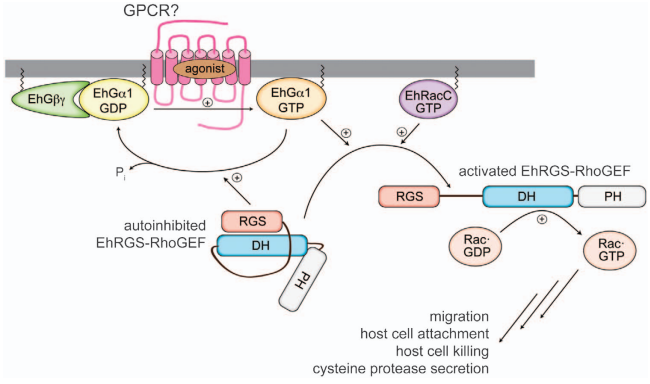

Figure 2 Model of heterotrimeric G protein signaling in *E. histolytica*. Activated EhGα1, together with guanosine triphosphate (GTP)-bound EhRacC, engages the autoinhibited EhRGS-RhoGEF (*E. histolytica* regulator of G protein signaling–Rho guanine nucleotide exchange factor) to promote Rac GTPase in *Drosophila* S2 cells,[63] although no specific *E. histolytica* Rho family substrate for EhRGS-RhoGEF has yet been identified. Both EhGα1 and EhRGS-RhoGEF alter trophozoite migration, host cell attachment and cell killing by altered cysteine protease secretion.[61,63] An associated G protein-coupled receptor (GPCR) is postulated but not yet established within this signaling pathway; Despite its name, the protein EhGPCR-1 is more likely a Wnt-binding factor than a ligand-activated heterotrimeric G protein GEF.[61] DH, Dbl homology; GDP, guanosine diphosphate; PH, pleckstrin homology.

effects on trophozoite function when compared with overexpression of dominant-negative EhGα1, consistent with the function of EhRGS-RhoGEF as a EhGα1 GAP (also demonstrated *in vitro*) and, thus, a negative regulator of heterotrimeric G protein signaling in the context of its overexpression.[61,63] Nucleotide exchange is rate-limiting in the EhGα1 nucleotide cycle,[61] as seen with mammalian Gα subunits, suggesting that GEF activity is needed for signal activation. Yet the *E. histolytica* genome lacks homologs of non-receptor GEFs for heterotrimeric G proteins such as Ric-8 and GIV,[30,64] leading to the hypothesis that *E. histolytica* may express one or more GPCRs (that is, a putative cell surface-spanning, EhGα1-directed GEF; Figure 2). Although a *bona fide* heterotrimeric GPCR has not yet been identified in this organism, one or more receptor/ligand pairs would provide valuable tools for manipulating G protein signal transduction in *E. histolytica* and also potentially serve as a candidate drug-discovery target.[36,61]

A second, putative Gα subunit (AmoebaDB acc. no. EHI_186910) exhibits a unique domain structure, with an N-terminal Gα-like fold easily identifiable despite substantial sequence divergence from mammalian Gα subunits and a C-terminal PP2C-related phosphatase domain.[62] The Gα-like region of this protein also lacks determinants for CTX- or PTX-mediated ribosylation (as does EhGα1); furthermore, this putative Gα subunit also lacks the otherwise very well-conserved nucleotide-binding motifs shared among all G proteins, suggesting a lack of nucleotide binding by this protein (unpublished data and Wittinghofer and Vetter[65]). This apparently expressed protein awaits functional assessment of its Gα-like domain and its unique relationship to the adjacent phosphatase domain.

EhGβ1 dimerizes with one of two *E. histolytica* Gγ subunits when expressed in mammalian cells, and the EhGβγ dimer in turn binds EhGα1 in a nucleotide state-selective fashion.[61] Gβγ subunits also frequently engage downstream effectors, even when the associated Gα subunits lack a major known effector, as seen in the cases of *Arabidopsis thaliana* sugar-sensing and yeast pheromone signaling.[66,67] Signaling downstream of EhGβγ is a distinct possibility for *E. histolytica* and may contribute to the phenotypic effects of perturbed EhGα1 expression;[61] however, no EhGβγ effectors have yet been identified.

RAS SUPERFAMILY GTPASES IN *E. HISTOLYTICA*

The *E. histolytica* genome encodes a remarkably large number of small GTPases for a single-celled parasite (>170 annotated in AmoebaDB[68]), suggesting a prominent role for Ras superfamily G protein signaling. The Ras superfamily can be divided into the Ras subfamily, typically regulating cell proliferation and survival; the Rho family that regulates actin organization, the cell cycle and gene expression; the Ran family, implicated primarily in nucleocytoplasmic transport; and the Rab and Arf families, known as regulators of vesicular transport and trafficking (reviewed in Wennerberg *et al.*[33]). Ten Ras proteins and two related Rap homologs have been described in *E. histolytica*,[69,70] although the complete Ras subfamily has not been enumerated since completion of the *E. histolytica* genome sequencing project. At least 20 Rho family GTPases, including Rho, Rac and Cdc42 homologs, are transcribed by *E. histolytica* trophozoites.[71–73] The Rab family is the most numerous small G protein group described in *E. histolytica*, with 91 annotated genes.[74] Although not yet described in the literature, putative Ran and Arf family GTPases also exist in the *E. histolytica* genome.[68] Although a small fraction of *E. histolytica* Ras superfamily GTPases has been investigated, the extent of functional redundancy, signaling specificity and nucleotide cycle regulation among these small G proteins remain largely unknown. Given the poor genetic tractability of *E. histolytica* trophozoites, investigations of G protein signaling in this organism have largely been limited to overexpression studies. Although overexpression is certainly an informative genetic perturbation, it should be noted that overexpressed G proteins, or nucleotide cycle-impaired mutants thereof, are subject to potential mislocalization and non-physiological functions.

Ras family GTPases

An initial study in *E. histolytica* trophozoites identified two *Ras* genes and two related *Rap* genes, as well as a single protein that apparently cross-reacted with a mammalian anti-Ras antibody.[69] Ras family GTPases in mammals and yeast are isoprenylated with either a geranylgeranyl or a farnesyl group at the characteristic C-terminal C*aa*X motif, where '*a*' is an aliphatic amino acid, and the final residue is predictive of either geranylgeranylation or farnesylation.[33] Expression of EhRap2, EhRas1 and C*aa*X motif mutants thereof in mammalian reticulocytes revealed that *E. histolytica* Ras GTPases can be isoprenylated, but that their C*aa*X motif sequences are less predictive of the specific isoprenyl group added than mammalian counterparts.[75] An *E. histolytica* farnesyltransferase, consisting of two subunits, was later cloned and shown to farnesylate human H-Ras and EhRas4 to the exclusion of three other *E. histolytica* Ras isoforms, indicating a distinct C*aa*X motif selectivity for isoprenylation.[70] Recombinant *E. histolytica* farnesyl transferase is resistant to mammalian farnesyltransferase inhibitors, precluding their use as tools in studying Ras GTPase function in *E. histolytica* trophozoites. Ras GTPases and related signaling machinery have been the targets of much pharmaceutical development effort, given the centrality of oncogenic Ras signaling to cellular proliferation and survival in many human malignancies.[76] However, no studies of perturbed Ras signaling in *E. histolytica* have yet emerged. Similarly, putative regulators of Ras nucleotide cycling (for example, GEFs and GAPs) and candidate Ras effectors are currently understudied in *E. histolytica*.

Rho family GTPases

E. histolytica possesses a highly dynamic, actin-rich cytoskeleton that participates in many pathogenesis-related processes (reviewed in Meza *et al.*[28]), as well as two major

actin-associated myosins (reviewed in Labruyere and Guillen[29] and Marion *et al.*[77]). Remarkably rapid actin remodeling is apparent in trophozoite motility,[78] a process regulated by extracellular matrix interactions[79] as well as self-generated chemokines.[80] Cytoskeletal remodeling is also intimately associated with *E. histolytica* phagocytosis[26] and surface receptor capping.[27] As master regulators of the actin cytoskeleton, as well as cell division and transcription in mammals, Rho GTPases and their associated proteins have been a focus of intense investigation in *E. histolytica*.

The first identified Rho family GTPase in *E. histolytica* was EhRho1 (Figure 3a), also later referred to as EhRhoA1.[71] As a homolog of human RhoA, EhRho1 was a natural candidate substrate for the Rho-inhibiting C3 exoenzyme from *Clostridum botulinum*, a protein whose ectopic expression in *E. histolytica* trophozoites leads to ribosylation of an ~25-kDa protein and reduces both proliferation and host cell killing.[81] However, recombinant EhRho1 was later found not to be a substrate for C3 exoenzyme,[82] but instead glucosylated *in vitro* by *Clostridum difficile* toxin B and *Clostridum novyi* α-toxin.[83] However, use of these two *Clostridium* toxins to study EhRho1 function *in vivo* is impaired by a lack of trophozoite membrane permeability.[83] A structural study of EhRho1 has more recently highlighted its conserved conformational difference between GDP- and GTP-bound states, as well as its distinct lack of a 'Rho insert helix'—a structural feature that differentiates all other Rho family GTPases from the greater Ras superfamily.[73] EhRho1 also differs from its mammalian homologs at a key nucleotide-binding residue, a feature found to confer rapid intrinsic nucleotide exchange but not constitutive activity.[73,82] However, EhRho1 does exhibit a signature activity of other Rho family GTPases; expression of a constitutively active mutant in human cells promotes actin stress fiber formation.[73] Activated EhRho1·GTP binds a Diaphanous-related formin effector protein, EhFormin1, to the exclusion of other *E. histolytica* Rho family GTPases (Figure 3a).[73,84] EhFormin1 is known to modulate actin polymerization, to be autoinhibited by an N- to C-terminal intramolecular interaction like its well-studied mammalian homologs,[85] and to be specifically activated by EhRho1·GTP.[84] A recent crystal structure of the EhRho1·GTPγS/EhFormin1 complex revealed a similar mode of intermolecular interaction as compared with mammalian counterparts, with the exception of a missing secondary binding site involving the Rho insert helix, and further yielded insights into specificity requirements for Rho GTPase/effector pairings.[84] EhFormin1 (also called EhDia) belongs to a family of eight *E. histolytica* formin proteins, three of which are Diaphanous-related (that is, containing tandem Rho GTPase-binding domains (GBDs) and formin homology 3 domains (FH3s)).[86,87] Overexpressed EhFormin1 in trophozoites localizes to pseudopodia, the microtubular assembly in the nucleus, and cytoplasmic F-actin structures in response to serum.[87] Furthermore, EhFormin1- and EhFormin2-overexpressing ameba exhibit cell division defects, with an increased number of nuclei per cell and increased average DNA content per nucleus,[87] suggesting that EhRho1/EhFormin1 signaling may be involved in actin polymerization in pseudopodia and/or trophozoite cell division (Figure 3a).

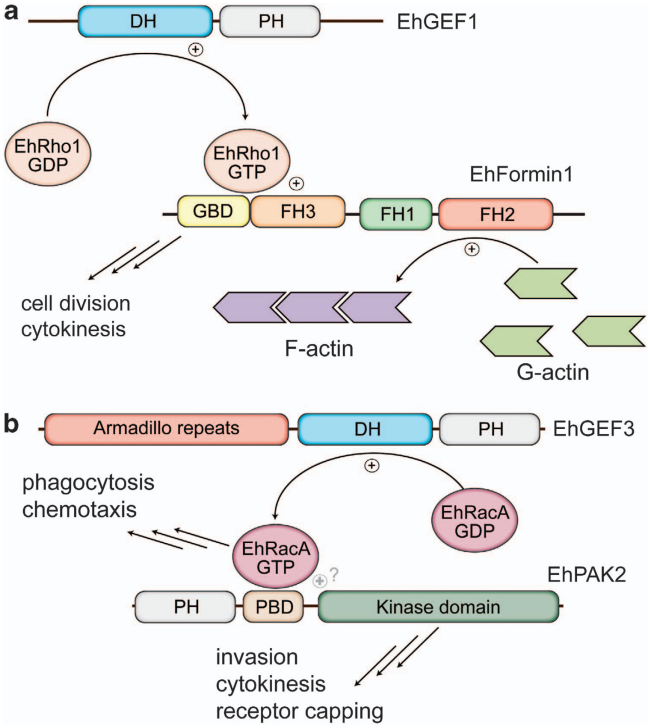

Figure 3 EhRho1 and EhRacA signaling modulate pathogenic behaviors in *E. histolytica*. (**a**) Nucleotide exchange on EhRho1 is known to be catalyzed by *E. histolytica* guanine nucleotide exchange factor 1 (EhGEF1) *in vitro*.[98] EhRho1 engages the GTPase-binding domain–formin homology 3 (GBD-FH3) domain tandem of the diaphanous-related and autoinhibited EhFormin1 to modulate actin polymerization.[84] EhFormin1 has also been implicated in trophozoite proliferation and cytokinesis.[87] (**b**) EhRacA nucleotide exchange is known to be accelerated by EhGEF3 *in vitro*.[102] Constitutively active EhRacA perturbs phagocytosis and chemotaxis, as well as surface receptor capping in trophozoites.[89] EhRacA·guanosine triphosphate (GTP) was also shown to bind *E. histolytica* p21-activated kinase 2 (EhPAK2), a likely effector whose kinase domain is implicated in collagen matrix invasion, cytokinesis and surface receptor capping.[90] Stimulation of EhPAK2 kinase activity by EhRacA is postulated but has not yet been established. DH, Dbl homology; GDP, guanosine diphosphate; PH, pleckstrin homology.

EhRho1 has also been implicated in signaling downstream of lysophosphatidic acid, an agent that promotes actin polymerization and associated F-actin structures, alters concanavalin A (ConA)-induced surface receptor capping, increases migration and invasion and modulates erythrophagocytosis by *E. histolytica* trophozoites.[54,88] Lysophosphatidic acid treatment (of the order of 10 μM concentration) has been reported to promote EhRho1 activation within *E. histolytica*, as measured by a GST (glutathione *S*-transferase)-Rhotekin Rho-binding domain pull-down assay.[54,88] However, we and others have been unable to observe nucleotide-specific interaction

between GST-Rhotekin Rho-binding domain and either epitope-tagged EhRho1 expressed in cells or purified recombinant EhRho1 (unpublished data), suggesting that EhRho1 binding observed in other studies[54,88] may be the result of non-specific interactions, or that the employed anti-EhRho1 antibody may cross-react with one or more other *E. histolytica* Rho family GTPases. Lysophosphatidic acid-induced EhRho1 activation has also been assessed by co-immunoprecipitation with a human antigen-derived anti-Rho kinase 2 antibody;[54] however no Rho kinase 2 homologs in *E. histolytica* have yet been described or are apparent in the genome.[68]

A number of Rac GTPase homologs are also expressed in *E. histolytica*,[72] including EhRacA. Overexpression of a constitutively active EhRacA(G12V) in trophozoites leads to delayed cell division, as well as defects in phagocytosis of bacteria, human erythrocytes and mucin-coated beads and alterations in ConA-stimulated receptor capping.[89] Furthermore, EhRacA was seen to specifically engage the p21-binding domain of the p21-activated kinase (PAK) EhPAK2, both in amebic lysates and in the context of purified recombinant proteins (Figure 3b).[90] PAKs are effectors for canonical Rho family GTPases, and their serine/threonine kinase activities and/or localizations are modulated by the binding of activated G proteins (reviewed in Kumar *et al.*[91]). Trophozoites engineered to overexpress the kinase domain of EhPAK2, but not the full-length protein or the N-terminal regulatory region, exhibit defects in collagen matrix invasion, surface receptor capping and cytokinesis.[90] Phenotypic overlap between EhRacA(G12V) and EhPAK2 kinase domain strains suggests a role for EhRacA/EhPAK2 signaling in surface receptor capping and regulation of cell division.

EhRacG has also been identified as a contributor to pathogenesis-related functions in *E. histolytica*. Overexpression of constitutively active EhRacG(G12V) in trophozoites leads to formation of a minor population of giant multinucleated cells, indicating a likely cytokinesis defect.[92] Filamentous actin arrangements and surface receptor capping are also altered with EhRacG(G12V) expression, and electron microscopy observations suggest increased budding of membrane vesicles.[92] Endogenously expressed EhRacG is enriched in ConA-induced uroids, together with filamentous actin and myosin II, consistent with its regulatory role in surface receptor capping *via* modulation of the actin cytoskeleton.[92]

Activated EhRacC was recently shown to directly engage the heterotrimeric G protein effector EhRGS-RhoGEF.[63] Expression of constitutively active EhRacC, together with constitutively active EhGα1 is required to achieve EhRGS-RhoGEF activation in *Drosophila* S2 cells,[63] suggesting a convergence with heterotrimeric G protein signaling (Figure 2). However, the contributions of EhRacC to cellular processes in *E. histolytica* remain to be directly assessed.

A number of other putative Rho family GTPase effectors have been described in *E. histolytica*, although without unequivocally associated G proteins. For instance, two other diaphanous-related formins with GBD-FH3 domain tandems are encoded by the *E. histolytica* genome in addition to the EhRho1 effector EhFormin1.[87] Overexpressed EhFormin2 in trophozoites, like EhFormin1, is localized in pseudopodia and pinocytic and phagocytic vesicles, and results in mitosis and cytokinesis defects,[87] suggesting some functional redundancy among diaphanous-related formins despite differences in their Rho-GTPase binding sites and, thus, likely differences in Rho activator specificities (Bosch *et al.*[84] and unpublished data). A fourth GBD-FH3 tandem protein, the actin-binding EhNCABP166, has also been implicated as a modulator of phagocytosis, chemotactic migration and possibly proliferation in trophozoites.[93] The small G protein specificity of the EhNACAP166 GBD-FH3 domain tandem has been investigated; however, these binding experiments were conducted with denatured Rho GTPases,[93] and intact Rho tertiary structure is required for the typical Rho/GBD-FH3 association (for example, Bosch *et al.*[84]). Some of the seven identified PAK family members, in addition to the EhRacA effector EhPAK2, have also been studied in *E. histolytica*. EhPAK (also called EhPAK1) localizes to pseudopods during amebic migration and to the uroid upon ConA-induced capping.[94] The N-terminus of EhPAK1 was found to bind human Rac1 with typical nucleotide specificity (that is, dependent on the GTP-bound state) despite the lack of an identifiable p21-binding domain; trophozoites overexpressing the EhPAK1 kinase domain exhibit reduced migration, an increased number of membrane extensions and an increased rate of erythrocyte phagocytosis.[95] EhPAK3 is also expressed in trophozoites, and both recombinant EhPAK3 purified under denaturing conditions and EhPAK3 immunoprecipitated from amebic lysates exhibit apparent kinase activity.[96]

Putative regulators of Rho family GTPase nucleotide cycling are also prominent in the *E. histolytica* genome,[30] including ~70 Dbl homology (DH) domain-containing candidate RhoGEFs, ~70 encoded RhoGAP domain-containing proteins and a single RhoGDI (EhRhoGDI; for example, Bosch *et al.*[73]). Although no studies of RhoGAP proteins have yet emerged, they are likely to regulate pathogenesis-related functions like their associated GTPases. Recombinant purified EhRhoGDI binds EhRho1 in a nucleotide state- and isoprenylation-dependent fashion.[73] As the only apparent RhoGDI, it is likely that this protein also engages other inactive Rho GTPases in *E. histolytica* to impair nucleotide exchange and regulate their subcellular localization.

Better studied are a number of Dbl family RhoGEFs. For example, overexpression of EhGEF1 in trophozoites decreases total cellular filamentous actin, reduces amebic migration and alters killing of mammalian cells.[97,98] *In vitro* nucleotide exchange assays indicate that EhGEF1 likely catalyzes exchange on EhRacG and EhRho1 (the latter illustrated in Figure 3a), although concentrations of GEF protein employed in these assays as well as a concentration–response analysis were not included in this report.[98] Later studies have used structural homology models to predict EhGEF1 DH domain

point mutations that impair GEF activity toward EhRho1 and EhRacG, as indicated by maximal nucleotide analog fluorescence at a single time point.[99] However, kinetic analysis is a preferable measure of GEF activity, as maximal fluorescence readings are subject to artifacts due to differing specific activities of recombinant proteins, non-specific binding, fluctuations in instrumentation settings, and/or 'buffer shifts' in fluorescence that vary among protein preparations. EhGEF1 small-molecule inhibitors have also been pursued, based on a docking analysis using a homology model to existing mammalian RhoGEF structures ($\sim$50% or less sequence similarity).[100] Five compounds were assessed for EhGEF1 inhibition by *in vitro* nucleotide exchange assays and found to be active at $\sim$50–100 μM concentrations.[100] However, exchange kinetics were not assessed, a typical concentration–response pattern was not obtained and direct binding of compounds to EhGEF1 (or potentially Rho GTPases) has not yet been demonstrated in these studies. Furthermore, the specificity of these potential pharmacological tools, for instance across other *E. histolytica* RhoGEFs, remains to be determined.

The Armadillo-repeat containing EhGEF2 has been implicated in erythrocyte phagocytosis, trophozoite proliferation and chemotaxis, based upon an *E. histolytica* strain engineered to overexpress a dominant-negative point mutant.[101] Both the N-terminal and DH domain regions were seen to contribute to EhGEF2 membrane localization. EhGEF2 was also suggested to activate EhRacA-D, EhRacG-H and EhCdc42 *in vitro*,[101] although no kinetic analysis was provided in this report and the fluorescence time courses shown appear to be caused by buffer shifts upon GEF addition rather than a single exponential binding event *per se*. Which Rho substrates and dominant-negative mutant-impaired signals are relevant for the observed *in vivo* effects are currently unknown.

The DH-PH (pleckstrin homology) domain tandem of a third Dbl family RhoGEF, EhGEF3, stimulates nucleotide exchange on EhRacA and EhRho1 *in vitro*.[102] Simultaneous EhGEF3 and EhRacA overexpression in *E. histolytica* leads to increased migration toward fibronectin, whereas a dominant-negative EhGEF3 mutant has the opposite effect. Overexpressed EhGEF3, but not the dominant-negative point mutant, also promotes EhRacA activation in trophozoites, as assessed by a GST-EhPAK2 p21-binding domain pull-down assay, suggesting a role for EhGEF3/EhRacA signaling in chemotactic migration (Figure 3b).[102] EhGEF3 and EhRacA co-localize in caps induced by ConA treatment, suggesting a possible additional role in surface receptor capping.[102]

Members of a family of 11 RhoGEFs in *E. histolytica* each contain a FYVE domain, known to associate with inositol phospholipids and to decorate early phagosomes in trophozoites.[103] A GFP-tagged mammalian FYVE domain overexpressed in trophozoites was observed to translocate to phagocytic cups and phagosomes during host cell phagocytosis.[104] One overexpressed FYVE domain-containing RhoGEF, EhFP4, is also recruited to phagocytosis-related structures,[104] and overexpression of the isolated FYVE domain from EhFP4 impairs trophozoite phagocytosis. Interaction of EhFP4 or its DH-PH domains with recombinant *E. histolytica* Rho family GTPases has been assessed with pull-down assays. EhFP4 was seen to interact with EhRacC, EhRacD, and two unnamed G proteins, although nucleotide state selectivity was not assessed in this study, and the authors reported inability to detect nucleotide exchange activity.[104] Thus, it remains to be established whether these FYVE domain-containing RhoGEFs exhibit GEF activity and whether there is any functional interplay between their FYVE and DH-PH domains.

Rab family GTPases

The *E. histolytica* genome encodes a remarkable 91 Rab family G proteins, many of which are not clear homologs of mammalian Rabs, suggesting an unusually high degree of complexity underlying vesicular trafficking regulation in trophozoites.[74,105,106] Endosomes isolated from *E. histolytica* by magnetic fractionation are associated with virulence-associated cysteine protease activity, as well as enrichment of Rab GTPases, such as EhRab11 and potentially a Rab7 homolog.[107] The importance of phagocytosis and pinocytosis to nutrient uptake by trophozoites, the secretion of virulence factors like amebapores and cysteine proteases, as well as the critical role of membrane-associated proteins like the Gal/GalNAc lectin to pathogenic behaviors, all support the hypothesis that Rab-regulated vesicular trafficking is important for *E. histolytica* biology and pathogenesis.[14,23,24]

EhRabA is localized to vesicles at steady state, but moves to the leading edge in motile cells and the membrane opposite ConA-induced caps, as well as to membrane extensions upon N-formyl peptide-induced polarization.[108] Expression of a dominant-negative EhRabA mutant in trophozoites produces changes in cell morphology and polarization, impairs motility and reduces host cell attachment and killing but has no observable effect on pinocytosis or erythrophagocytosis.[109] Conversely, overexpression of constitutively active EhRabA perturbs erythrophagocytosis and leads to formation of large tubular organelles apparently derived from the endoplasmic reticulum (ER).[110] Two subunits of the Gal/Gal-NAc lectin and a cysteine protease are mislocalized to these EhRabA-induced organelles, and similar effects are seen with brefeldin-A treatment, suggesting that EhRabA regulates trafficking between the ER and Golgi apparatus in *E. histolytica*.[110]

EhRabB is one of the first identified and most frequently studied Rab family GTPases in *E. histolytica*. Initial immunofluorescence studies localized endogenous EhRabB to cytoplasmic vesicles and noted its translocation to the plasma membrane and phagocytic cups during erythrophagocytosis.[111,112] Poor phagocytosis in a mutant *E. histolytica* strain was seen to correlate with increased expression of EhRabB, as well as substantial sequence differences between this mutant EhRabB and wild-type EhRabB, providing further evidence for its involvement in phagocytosis,[113] although a causal association was not established. EhRabB was also observed to

be enriched at phagosomes in a proteomics study.[114] Overexpression of wild-type EhRabB in trophozoites leads to a small diminution of phagocytosis, while expression of a dominant-negative mutant (N118I) leads to decreases in both phagocytosis and cell monolayer destruction.[115] Of particular interest, EhRabB(N118I)-expressing trophozoites do not form liver abscesses in a hamster model, while vector-transfected and wild-type EhRabB-expressing amebae do form such abscesses, establishing EhRabB signaling as likely important for pathogenesis.[115] EhRabB was reported to interact with the transmembrane protein EhGPCR-1 by yeast two-hybrid, although binding data were not shown in this study.[116] Despite its initial naming as a GPCR, further sequence analysis has indicated that EhGPCR-1 is more likely a Wnt-binding factor rather than a ligand-activated heterotrimeric G protein GEF *per se*.[61]

E. histolytica also expresses Rab5- and Rab7-related G proteins.[117,118] Overexpressed EhRab5 and EhRab7A both localize to independent vesicular structures at steady state, but exposure of these overexpression-modified trophozoites to erythrocytes is seen to cause convergence of the two Rabs at a large 'pre-phagosomal vacuole', distinct from actual phagosomes.[119] Electron microscopic studies have identified small amebapore-containing vesicles in the pre-phagosomal vacuole, suggesting a role for EhRab5 and/or EhRab7A in delivering cytotoxic amebapores to phagosomes.[119] Consistent with this hypothesis, overexpression of wild-type EhRab5 enhances phagocytosis kinetics and amebapore transport, while expression of either constitutively active or dominant-negative EhRab5 mutants impairs pre-phagosomal vacuole formation and phagocytosis.[119] EhRab7 also co-localizes with early endosomes,[117] and overexpression of EhRab7A in trophozoites reveals its subcellular localization to lysosomes and an increased acidic cellular compartment as well as decreased cellular cysteine protease activity.[120] A retromer-like complex of *E. histolytica* proteins is seen to engage recombinant EhRab7A in a nucleotide-dependent fashion, primarily through the C-terminus of EhVps26, leading to the hypothesis that EhRab7A may contribute to retrograde transport from vacuoles and phagosomes to the Golgi apparatus.[120] An *E. histolytica* homolog of Rab8 has also been cloned, but no cellular functions have yet been established for this G protein other than its vesicular localization.[121,122]

EhRab11 exhibits a punctate distribution in trophozoites and moves to the cell periphery upon iron and serum starvation of trophozoites,[123] in contrast to EhRab7 and EhRabA. Iron and serum starvation is associated with altered cytokinesis and increased detergent-resistant cells, but whether EhRab11 contributes to these phenotypes is unknown.[123] A related isoform, EhRab11B, also exhibits a vesicular distribution, and overexpression of EhRab11B in trophozoites leads to an increase in both intracellular and secreted cysteine proteases.[124] Amebae overexpressing EhRab11B exhibit slightly increased exocytosis of a fluid-phase marker and more efficiently kill mammalian cells, an effect reversed by treatment with the cysteine protease inhibitor E64.[124] These findings suggest that the *E. histolytica* Rab11 isoforms have non-redundant functions.

No studies of Rab GTPase nucleotide cycle regulators in *E. histolytica* have yet emerged. The *E. histolytica* genome encodes for ~20 proteins with DENN (after 'differentially expressed in neoplastic vs normal' cells) domains,[68] known in mammals to serve as Rab GEFs, along with other structurally unrelated proteins.[125] Also present are ~50 Rab-GAP/TBC (Tre-2, Bub2, and Cdc16) domain-containing proteins and two putative Rab GDIs.[68] Examination of these likely Rab regulators may shed further light on signaling mechanisms contributing to *E. histolytica* pathogenicity, especially in the context of vesicular trafficking mechanics.

CONCLUSION AND PERSPECTIVE

A number of G proteins have been implicated in key pathogenic processes of *E. histolytica*, particularly heterotrimeric G proteins, a number of cytoskeleton-associated Rho GTPases, and also Rab GTPases primarily involved in vesicular trafficking. Exploitation of known signaling pathways for pharmacological manipulation is attractive, both in the development of tools for interrogating the specific functions of G protein signaling in *E. histolytica* and as a potential approach to the development of anti-amebiasis therapeutics. A first step has been taken in developing small molecule inhibitors for EhGEF1,[100] and other *E. histolytica* RhoGEFs may be targetable given some previous success in inhibiting mammalian Rho GTPase activation (for example, Shutes *et al.*[126] and Evelyn *et al.*[127]). Some Ras and Rho GTPase effectors, particularly kinases like the PAKs and members of the mitogen-activated protein kinase cascade, have also proven tractable as pharmacological targets in humans (for example, Rusconi *et al.*[128] and Zhao and Manser[129]). However, the importance of Ras effectors and downstream kinases in *E. histolytica* pathogenesis has not yet been explored. Particularly promising for pharmacological development is the recently described heterotrimeric G protein signaling within *E. histolytica*,[36,61] although identification of a *bona fide* GPCR and ligand pair in *E. histolytica* remains a barrier at this time.

Aside from pharmacological goals, much remains to be discovered regarding the modulation of Ras superfamily GTPase functions in *E. histolytica*, particularly regarding nucleotide cycle regulators (GAPs, GEFs and GDIs). Also unclear is the interplay among the well-populated small G protein families in *E. histolytica*, such as the >20-member Rho family and the 91-member Rab family. Are many of these GTPases redundant in function and regulation? How is GTPase specificity for effectors and nucleotide cycle regulators achieved, given such large numbers of simultaneously expressed G proteins in a single cell? Further study of both heterotrimeric and small G proteins in *E. histolytica* will likely add to our understanding of parasite biology and pathogenicity, as well as signaling in other organisms.

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